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#### (57) Abstract

The present invention provides a novel family of organic anion transporters of which until now only one member was known. The family includes multispecific organic anion transporters related to the canalicular multispecific organic anion transporter. The cDNA encoding the latter is also provided. The rat and human cDNA are exemplified. Uses of nucleic acids based on this gene family and of cells comprising such nucleic acids as well as vectors comprising sequences thereof are also disclosed especially in the area of gene therapy.

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Title: A family of organic anion transporters; nucleic acids encoding them, cells comprising them and methods for using them

The present invention lies in the field of molecular biology and genetic engineering. It is particularly concerned with mechanisms of transport for substances across cell membranes. More in particular it is concerned with transport of cytotoxic substances from the inside to the outside of cells.

A group of proteins involved in transport of molecules across membranes is the group of so-called ABC-transporters (ABC:  $\underline{ATP-b}$ inding  $\underline{c}$ assette). One member of this group called 10 MRP1 (multidrug resistance-associated protein) has been identified as being involved in transporting organic anions across cell membranes. This protein thus transports different substances than the P-glycoprotein encoded by the MDR-1 gene (MDR: multidrug resistance). MDR-1 is involved in 15 the occurrence of multidrug resistance of for instance tumor cells. Multidrug resistance is one of the major problems in chemotherapy of cancer. On the other hand providing cells with multidrug resistance may be very useful in rescuing for instance bone marrow when chemotherapy is applied. Thus on the one hand there is a need for being able to prevent 20 transport of cytotoxic substances out of the cell while on the other hand there is a need to be able to enhance transport of cytotoxic substances from cells. The Pglycoprotein encoded by MDR-1 is not capable of transport of 25 all cytotoxic substances; its binding specificity is limited to certain groups of molecules. MRP-1 has a different binding specificity in that it transports anionic organic compounds, possibly complexed or conjuged with other substances. In the liver a protein has been characterized 30 functionally which is an ATP-dependent non-bile salt organic anion transporter called canalicular Multispecific Organic Anion Transporter. This protein has been thought to be identical to MRP-1.

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The liver plays a major role in the detoxification of many endogenous and xenobiotic, lipophilic compounds. Detoxification is accomplished by transferase-mediated conjugation with glutathione-, glucuronide-, or sulphatemoieties, resulting in negatively charged, amphiphilic 5 compounds which are efficiently secreted into bile or urine. Hepatobiliary excretion of these conjugates is mediated by an ATP-dependent transport system, the canalicular Multispecific Organic Anion Transporter (cMOAT), located in the apical (canalicular) membrane of the hepatocyte (1). The 10 identification of a transport-deficient mutant rat strain, the TR rat (2), has contributed to the functional characterization of cMOAT (1). These rats have an autosomal recessive defect in the hepatobiliary excretion of bilirubin glucuronides (3) and other multivalent organic anions 15 including, glutathione-S-conjugates (e.g. leukotriene  $C_4$ ), and 3-OH-glucuronidated and -sulphated bile salts (4). Thus far neither a protein nor a complete cDNA encoding cMOAT have been identified. Transport studies in plasma membrane vesicles from cells overexpressing the human Multidrug 20 Resistance-associated Protein 1 (hMRP1) (5), demonstrated a role for hMRP1 in the ATP-dependent transport of the glutathione conjugates  $LTC_4$  and dinitrophenyl glutathione (GS-DNP) (6). Because these substrates are also transported by the putative cMOAT protein, MRP1 as stated before, is a 25 possible candidate gene for cMOAT. A recent study suggested lateral as well as canalicular localization of the rmrpl gene product in Wistar liver, but only a lateral localization in TR liver (14) and suggested a role for MRP1 in the (defective) hepatobiliary excretion of organic anions 30 in TR rats. In our view, however, the extremely low MRP1 expression in liver (7,5) renders it unlikely that this gene product is responsible for biliary organic anion secretion. Furthermore, the transport defect in the TR rat appears to be specific for liver (9), while MRP1 is expressed in all 35 human tissues (7). We have now found that cMOAT is encoded

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by a different gene and thus that a family of organic anion transporters exists.

The present invention now provides a nucleic acid comprising a sequence encoding at least a part of a member of a family of organic anion transporters, said nucleic acid comprising at least a gene family specific fragment of one of the sequences of fig.la or fig.lb or figs 17, 18 or 19 or the complement thereof, or a sequence having at least 55%, preferably 70%, in particular 90% homology therewith. Of this family sofar only one member, mammalian MRP was known.

We hypothesized that cMOAT might be a liver-specific homologue of MRP1. To obtain a rat mrp1 probe, we applied the polymerase chain reaction (PCR) on rat lung cDNA using nested degenerate oligonucleotide primers which were based on the highly conserved first ATP-binding cassette of the 15 hMRP1 sequence (see experimental part). The 213 base pair product obtained shared 83% amino acid sequence identity with the corresponding region of the hMRP1 sequence. When analyzed on Northern (RNA) blot, this PCR fragment 20 hybridized with a single, 9.5-kb, transcript in all Wistar and TR rat tissues examined, with high expression in lung and testis, but no detectable expression in liver. Because this expression pattern resembled that of hMRP1 in human tissues (7), we assumed that we had isolated a part of the 25 rat homolog of hMRP1, rat mrp1 (rmrp1). In order to find the putative cmoat gene, two rat liver cDNA libraries were screened, using the rmrp1 fragment obtained as a probe (see the experimental part). This resulted in the isolation of a full length cDNA with a single open reading frame of 1541 30 amino acids (Fig. 1b). Based on similarity searches (10), the protein was identified as a new member of the ABCtransporter family (11), with modest identity to other members of the family. Highest overall identity was found with hMRP1 (47.6%) (5), yeast Cadmium Factor 1 (41.8%) (12), and the human Cystic Fibrosis Transmembrane Conductance Regulator (30.2%) (13). The amino acid sequence identity

with hMRP1 ranged from 38-61% outside the ATP-binding

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domains to 67% and 75% in the first and second ATP-binding domain, respectively. Recently, two different partial rmpl cDNA sequences were disclosed (14). One of these sequences comprised a 347 nt fragment that closely resembles the rat cmoat cDNA sequence found by us, said partial sequence is, however, not identical to cmoat. Moreover, no relation has been made between said partial sequence and the putative cMOAT protein. In contrast, it was suggested that a mutant mrpl gene is responsible for the cMOAT-deficient phenotype.

Northern (RNA) blot analysis of rat tissues with a 1-kb restriction fragment of our isolated cDNA, revealed three different transcripts, ranging from approximately 6.5 to 9.5-kb, with high expression only in liver, and low expression in kidney, duodenum, and ileum (Fig. 2A). These transcripts were strongly decreased (but not absent) in liver (Fig. 2B) and other tissues of the TR<sup>-</sup> rat, which suggests that these transcripts were related to the defect in the TR<sup>-</sup> rat. The three transcripts observed were probably derived from a single gene, because the level of all three transcripts was decreased in the TR<sup>-</sup> rat. The decrease of this transcript in TR<sup>-</sup> liver suggests that the isolated cDNA encoded cmoat.

To examine the expression level and the cellular localization of the cMOAT protein in hepatocytes, we produced a monoclonal antibody (mAb M<sub>2</sub> III-5) to a bacterial fusion protein containing the 202-amino acid carboxylterminal end of the sequence (see experimental part). On protein blots this antibody detected a protein of approximately 200-kD in the canalicular, but not the basolateral plasma membrane fraction of the Wistar rat liver (Fig. 3). This molecular weight was very similar to that of hMRP1 and in good agreement with the predicted molecular weight of the cMOAT protein. The 200-kD protein was completely absent from the canalicular membrane fraction of the TR<sup>-</sup> rat (fig.3), which correlated with the decreased mRNA level in TR<sup>-</sup> rat liver (Fig. 2B). Again, this finding was in good agreement with the defect observed in TR<sup>-</sup> rats

BNSDOCID: <WO\_\_\_\_\_9731111A2\_I\_>

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which lack a functional transport system for organic anions in the canalicular membrane.

Thus we have isolated the complete cDNA encoding the cMOAT protein, which is deficient in the TR rat. Since the cmoat mRNA was not completely absent in TR liver it was possible to also amplify the complete TR- cmoat cDNA by PCR (see the experimental part) using various specific primer sets. To identify the nature of the genetic defect in TRrats, we sequenced the obtained cDNA. This revealed a 1-bp deletion at amino acid position 393, which results in a frame-shift and subsequent introduction of a stop-codon at position 401 (Fig. 4). This deletion results in the destruction of a NlaIII restriction site ./hich provided a means to quickly confirm the mutation in cDNAs from various tissues (see the experimental part). The very low mRNA expression in TR rats (Fig. 2B) might be due to the fact that the frame shift causes premature termination of translation and subsequent increased degradation of the mRNA.

20 Our results show a correlation between the cmoat gene, the absence of the gene product from the canalicular membrane, and the defined congenital transport defect in TRrats. In addition to the exclusive canalicular localization of cMOAT (Fig. 3), we have found that hMRP1 is routed only 25 to the lateral domain of the plasma membrane of pig kidney epithelial cells (see experimental part). Our findings thus suggest a differential localization of MRP1 (basolateral) and cMOAT (canalicular) and imply that cMOAT and not MRP1 is involved in biliary organic anion transport. This contrasts 30 to suggestions made in the literature (14). It was also suggested (14) that an isoform of MRP1 exists in rat liver which is derived from the same gene by alternative splicing based on the detection of two different sequences for the second ATP-binding domain and only one for the first ATP-35 binding domain. Our complete cDNA data, however, show that there are also two different sequences for the first ATPbinding domain in mrpl and cmoat. In fact, the two cDNA

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sequences differ considerably throughout the entire molecule, thus indicating that MRP1 and cMOAT are encoded by two different genes.

We conclude that the MRP homolog, identified here, encodes the canalicular Multispecific Organic Anion Transporter, and that a 1-bp deletion, resulting in a truncated, non-viable, protein, is responsible for impaired transport of organic compounds from liver to bile in the TRrat.  $TR^-$  rats have the same phenotype as patients with the Dubin-Johnson syndrome, characterized by mild chronic 10 conjugated hyperbilirubinemia (15). Isolation of the human homolog of cmoat is required to elucidate the nature of the defect in humans. Overexpression of hMRPl confers resistance of human tumor cells to a number of cytostatic drugs (16, 17), and this resistance is dependent on intracellular 15 glutathione levels (18). Apparently, both MRP1 and cmoat are involved in the excretion of organic anions from cells. Thus, overexpression of cMOAT, like that of MRP1, might also confer resistance to cancer cells against cytostatic drugs or their metabolites. 20

Using the rat *cmoat* gene we also found and isolated the cDNA encoding the human cMOAT protein. Now that it is known that these two exist other species of this family of transporters can be found using the present invention.

These transport mechanisms occur throughout the living world, so family members can be found in bacteria, bacilli, yeasts and fungi, plants, invertebrae, vertebrae, in particular in mammalians.

In addition to MRP1 and cMOAT (MRP2), other MRP homologs encoding GS-X pumps are present in the human genome, considering that there are at least four MRP homologs expressed in Caenorhabditis elegans (56). We therefore searched the Expressed Sequence Tag (EST) library (57) for putative human MRP homologs, and found three more MRP homologs expressed in humans. We call these new MRP homologs MRP3, MRP4, and MRP5.

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To investigate a possible role of MRP homologs in drug resistance, we examined a large set of (multi)drug resistant cell lines for the (over)expression of cMOAT, MRP3, MRP4, and MRP5. We find that especially cMOAT expression is elevated in several cell lines, selected for cisplatin resistance, and also in some sublines of the human non-small lung cancer cell line SW1573/S1, selected for doxorubicin resistance. The expression level of cMOAT correlates with the cisplatin but not the doxorubicin resistance of these cell lines. Although MRP3 and MRP5 were overexpressed in some resistant cell lines, no clear correlation between drug resistance and the expression levels of MRP3, MRP4, and MRP5 has emerged from these studies as yet.

Preferred for the purposes of this invention are closely related members of the members identified by the sequences of fig.la and fig.lb, most preferred those members which transport similar or the same compounds when expressed in a cell, or the closely related family members identified herein as MRP 3, 4 and/or 5. Most preferred is the human cmoat gene or its human family members and their products for their usefulness in for instance gene therapy and for their use in preparing blocking agents to the transporting product.

Further embodiments include but are not limited to a vector comprising a nucleic acid according to the invention and suitable means for replication, transduction and/or expression of said nucleic acid.

Preferably such a vector further comprises a gene encoding a therapeutically beneficial protein, which may be any protein having a beneficial effect under certain circumstances such as giving glutathion elevating activity, which enhances transport of anionic complexes or conjugates by the invented transporters.

Such vectors include vectors wherein the gene encodes 35 at least a functional part of a gamma glutamyl cysteine synthetase or a UDP-glucuronosyltransferase.

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Other vectors according to the invention include vectors wherein the therapeutically beneficial protein is another multidrugresistance related protein such as MDR1.

The invention further provides a cell comprising a nucleic acid or a vector according to the invention. Said cells may be any cells, preferred are bone marrow progenitor cells, in particular hematopoietic stem cells.

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If said vector thus not encode additional desired functionalities apart from the cMOAT activity as disclosed above, said activity may be present on a separate vector to be introduced into said cell.

The invention also provides a method for providing cells with Canalicular Multispecific Organic Anion Transport protein activity, comprising transducing said cell with a nucleic acid or a vector according to the invention, as well as a method for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according to the invention, comprising increasing the intracellular level of glutathion, glucuronide and/or sulphate. This may be done by contacting the cell with for instance glutathion esters, but also by providing additional genetic material as disclosed above. This may be done by cotransducing UDP-glucosedehydrogenase or sulphotransferase or any other means of enhancing such activity.

Thus the invention also encompasses methods for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according to the invention, comprising enhancing the conjugating capacity and/or the complexing activity of said cell for sulphate, glutathion, glucuronide and the like.

On the other hand the invention provides a method for reducing Canalicular Multispecific Organic Anion Transport protein activity and/or the multidrug resistance of a cell comprising providing said cell with an antisense construct of a nucleic acid or a vector according to the invention, which antisense constructs are thus also part of the present invention. These methods can be used to block or at least

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reduce transport of substances by the transporter protein according to the invention thus reducing resistance of for instance tumor cells to certain chemotherapeutic substances. Other ways of blocking the invented transporter are also part of the invention. These include methods of reducing the level of the conjugating or complexing molecules that enhance transport by the invented transporter. Antibodies to (in particular the extracellular domain of) the transporter.

For even further reducing multidrug resistance of for instance tumor cells said cells can be further provided with an antisense construct derived from another multidrug resistance related protein such as MDR1.

Proteins encoded by a nucleic acid according to the invention or obtainable by expression of a vector according to the invention are of course also part of the present invention, in particular proteins having Canalicular Multispecific Organic Anion Transport protein activity or Canalicular Multispecific Organic Anion Transport protein specific antigenicity comprising at least part of the sequence of fig.4 or being encoded by at least a part of the sequences of MRP 2, MRP 3 or MRP 4 (as given in the accompanying figures) or derivatives thereof having the same or similar function.

In the following a number of uses of the molecules, cells and methods of the invention are disclosed.

The invention enables the use of a nucleic acid according to the invention or a protein according to the invention in the diagnosis of Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport protein, as well as the use of a nucleic acid according to the invention or a protein according to the invention in the treatment of Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport protein.

Furthermore the nucleic acids according to the invention can be used as a selectable marker gene.

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The members of the gene family disclosed herein have several useful applications in the context of gene therapy.

The concept of gene therapy has a very broad range of applications with one common denominator and that is the transfer of additional, new or corrected genetic information into cells which have a genetic or acquired defect. Examples of genetic disorders eligible for gene therapy are cystic fibrosis, Duchenne's Muscular Dystropy, cancer, Gaucher disease, Crigler Najjar and Dubin-Johnson syndrome. Examples of acquired diseases are cancer, viral and parasitic 10 diseases. In addition, gene transfer can augment the efficacy of conventional therapies. Vehicles for the transfer of genes into target cells and tissues include vectors of viral and non-viral origin. Among the viral vectors murine based retroviruses and human based 15 adenoviruses are the preferred embodiments.

Retroviruses are RNA viruses which efficiently integrate their genetic information into the genomic DNA of infected cells via a reverse-transcribed DNA intermediate as a proviral copy. Integration into the host's genome and the fact that parts of their genetic material can be replaced by foreign DNA sequences make retroviruses one of the more lucrative vectors for gene delivery in human gene therapy procedures, most notably for gene therapies which rely on gene transfer into dividing tissues. Recombinant murine retroviruses have been the vectors of choice since the start of gene therapy and several clinical trials using recombinant retroviruses are ongoing. In order to generate a recombinant retrovirus which carries the cDNA sequence of a particular gene one needs to introduce the retroviral construct into an appropriate packaging cell line. The retroviral construct carries the cDNA of interest and the cis acting elements for packaging and transcription of the viral RNA genome. The packaging cell line provides the trans acting factors needed for packaging: the gag, pol and env genes. Expression of the retroviral construct into the

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packaging cell line results in the production of recombinant retroviral particles capable of transducing susceptible target cells and transferring a particular therapeutic gene. The recombinant retrovirus is stably integrated into the target cell genome and conferred to its daughter cells upon cell division.

Adenoviruses are non-enveloped DNA viruses. The genome consists of a linear, double stranded DNA molecule of about 36 kb. Recombinant adenovirus vectors have been generated for gene transfer purposes. Recombinant adenoviruses can be generated by co-transfection of two El-deleted recombinant adenoviral DNA constructs, one of which comprising the sequences of interest, into an El-expressing cell line. In contrast to retroviruses, adenoviruses do not integrate into the host cell genome, are able to infect non-dividing cells and are able to efficiently transfer recombinant genes in vivo. These features make adenoviruses attractive candidates for in vivo gene transfer into target cells which are difficult or impossible to treat ex vivo, such as cells of lung and liver.

Although the skilled artisan will be able to employ other vector systems than those exemplified here, such as Adeno Associated Virus (AAV), adenoviruses and retroviruses are preferred embodiments, because of the extensive experience with these viruses in gene therapy concepts.

Vectors comprising nucleic acids encoding and expressing functional members of the family of organic anion transporters disclosed in the present invention are of particular importance for the treatment of diseases caused by defects in these transporters. Examples of such diseases include Dubin-Johnson syndrome, Rotor syndrome and other cholestatic disorders. The human Dubin-Johnson syndrome

The earliest evidence for distinct canalicular transport systems for bile acids and non-bile acid organic anions came with the recognition of the human Dubin-Johnson

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syndrome; this is a rare congenital chronic conjugated hyperbilirubinemia. The hepatic clearance of bilirubin and other cholephilic organic anions, like BSP and indocyanine green, is impaired in these patients, whereas bile acid clearance is normal. The urinary excretion of coproporphyrins, metabolic by-products of heme synthesis, is normal but the proportion of coproporphyrin isomer I is increased. The liver histology of this syndrome is characterized by lysosomal pigment accumulation.

Preferred target tissues for the genetic treatment of these diseases include the liver, gut and kidney.

Retroviral vectors comprising the nucleic acid sequences disclosed in this invention are constructed as exemplified in EP/95.201211.0 incorporated herein by reference. Recombinant retrovirus supernatant stocks are produced by introduction of the retroviral constructs in appropriate retroviral packaging cell lines. Adenoviral vectors comprising the nucleic acid sequences disclosed in this invention are constructed as exemplified in EP/95.202213.5 incorporated herein by reference. Adenovirus stocks are produced by transfecting the adenoviral construct into appropriate El complementing cell lines.

Hematopoietic stem cells (HSC) are the source for lifelong production of all mature blood cell types. Therefore, 25 genetic correction of HSC is expected to result in permanent mitigation of the clinical manifestation of inherited and aquired hematopoietic diseases. Of particular interest are Gaucher disease, thalassemia, sickle-cell anemia, AIDS, and others exemplified in WO93/07281. This makes HSC attractive 30 targets for gene therapy. Currently, the established procedure for gene transfer into long-term repopulating HSC relies on the use of recombinant retroviral vectors. However, the gene transfer efficiency into human HSC is insufficient for the treatment of most hematopoietic 35 diseases. This forms the bottle-neck for a broader application of bone marrow gene therapy. Therefore, it is

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preferred to provide the recombinant retroviral vector with a marker sequence for positive selection of transduced cells. Selection for the presence of this sequence can be performed in vitro by culturing the transduced cells in the presence of a selective drug. Another approach is to select for transduced cells in vivo, following transplantation of transduced HSC. Both approaches can be taken by inclusion in the recombinant retroviral construct genes encoding transporter proteins conferring resistance to cytostatic drugs.

The members of the family of organic anion transporters disclosed in the present invention are important examples of genes that can be used for this purpose.

15 Another important embodiment of the present invention is the use of the disclosed members of the family of organic anion transporters to provide the hematopoietic system of cancer patients with resistance to chemotherapeutic drugs. This makes increased dose-intensity in the chemotherapeutic 20 treatment of cancer possible. For most anticancer drugs increasing the dose-intensity results in increased response rates and a higher proportion of cures. Dose-intensity is the amount of drug administered per unit time, and can be augmented either by increasing the chemotherapy dose or by 25 reducing intervals between cycles. Dose-intensive chemotherapy can produce complete regressions and improve survival in patients with historically refractory solid tumors and non-Hodgkin's lymphomas.Dose-response relationships have been demonstrated for many anticancer 30 drugs. The major dose-limiting toxicity of many anticancer drugs is myelosuppression, which thus prevents optimum doseintensity administration. Severe myelosuppression makes the patient particularly prone to opportunistic infections and is a frequent reason for curtailing chemotherapy before an 35 adequate therapeutic response has been obtained. With a few exeptions (e.g., hormones) most anticancer drugs used in the clinic today, cause moderate to severe bone marrow toxicity

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(e.g., vinblastine, epipodophyllotoxins, cisplatin, carboplatin, melphalan, methotrexate, alkylating agents, nitrosoureas, anthracyclines and anthraquinones).

Increasing the therapeutic index of myelosuppressive anticancer drugs by retrovirus-mediated transfer of genes 5 encoding proteins conferring drug resistance is an attractive prospect. A recombinant retrovirus encoding a mutated dihydrofolate reductase (DHFR) that is highly resistant to the anticancer drug methotrexate has been constructed. Infection of murine bone marrow cells with this 10 retroviral vector and subsequent reconstitution of lethall; irradiated mice conferred protection from methotrexateinduced marrow toxicity. Furthermore, it has been demonstrated that transfer of the MDR1 cDNA into drugsensitive cells can introduce drug resistance, in vitro as 15 well as in vivo. Members of the family of drugs extruded from the cell by the MDR1 drug pump are e.g. anthracyclines, vinca alkaloids, podophyllotoxins, and colchicine. Etoposide, a commonly used podophyllotoxin of which the dose-limiting toxicity is restricted to the hematopoietic 20 system, is also pumped by the MDR1 encoded drug pump albeit only poorly. The MDR related drugs have in common that they are lipophilic compounds derived from various natural products. In general, MDR cells are not cross-resistant to alkylating agents (e.g., chlorambucil and cyclophosphamide), 25 antimetabolites (e.g., cytarabine, methotrexate, and 5fluorouracil), cisplatin, carboplatin or melphalan. The members of the family of organic anion transporters disclosed in the present invention efficiently extrude organic anion compounds from the cell, including GS-DNP and 30 chemotherapeutic agents such as the conjugated forms of cisplatin, carboplatin, etoposide, chlorambucil, and melphalan. This is shown in a nonlimiting example for cisplatin below. Therefore, introduction of the disclosed transporters into the hematopoietic system of cancer 35 patients allows dose-intensification of frequently used chemotherapeutic drugs such as etoposide and are different

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from those protected against by MDR1 or mutant DHFR. Therefore introduction of members of the disclosed invention into the hematopoietic system could lead to increased efficacy of cancer treatment.

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The invention will be described in greater detail in the following experimental part.

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#### Experimental

Example 1. Identification and isolation of the rat cmoat (mrp2).

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A 213-bp PCR product was obtained from rat lung cDNA after first round amplification with degenerate primers corresponding to amino acid residues 678-648 (forward) and 770-776 (reverse), and subsequent second round amplification with nested primers corresponding to amino acid residues 694-700 (forward) and 760-766 (reverse) of the hMRP1 sequence (5).

Partial cDNA clones were isolated from a rat hepatocyte cDNA library (23) which was screened with the 213-bp probe according to standard procedures (24). From a 4.5-kb 15 positive clone a 5'-located, 0.6-kb HphI restriction fragment was used to screen a gt10 5'-stretch rat liver cDNA library (Clontech, Palo Alto). A 0.8-kb overlapping clone was obtained from which a 0.6-kb AvaII probe was isolated to rescreen the same library, resulting in the 20 isolation of another overlapping clone. The 5' end of the cDNA was obtained using the anchored PCR procedure [M.A. Frohman, M.K. Dush, G.R. Martin, Proc. Natl. Acad. Sci. U.S.A. 85, 8998 (1988)]. cDNA synthesis was carried out with 5  $\mu g$  of total RNA isolated from Wistar rat liver and random 25 hexamer primers using Superscript Reverse Transcriptase II. After purification the cDNAs were tailed with a synthetic oligonucleotide anchor sequence using a 5'-RACE kit (Life Technologies, Gaithersburg). Two rounds of nested PCR (96°C, 30sec; 60°C, 30sec; 72°C, 45sec) using an anchor specific 30 primer and two cmoat-specific primers (5'tgtccagtatcttctgtgagcg-3'(first round), 5'aacacgacgaacacctgcttggc-3'(nested)) resulted in the isolation of the missing 5'-sequence. Probes were labeled with  $[a^{-32}P]dCTP$  using random primers. Hybridization of the 35 filters was performed at 65°C in 0.5 M NaPO<sub>4</sub> (pH 7.0), 2 mM EDTA, and 7% SDS (hybridization solution), for 20 hours.

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Filters were washed four times in 2x SSC, 1% SDS for 30 min at 65°C, and autoradiographed. Nucleotide sequences were determined by the dideoxy-nucleotide chain method [F.Sanger, S. Nicklen, A.R. Coulson, Proc. Natl. Acad. Sci. U.S.A. 74, 463 (1977)]. The *cmoat* sequence is being submitted to the Genbank database and is available under accession number L49379.

A fusion gene, consisting of the gene for the Escherichia Coli maltose-binding protein, and the 3' part of the cmoat cDNA corresponding to amino acid residues 1340-1541, was constructed in pMal-c [C.V. Maina et al., Gene 74, 365 (1977)]. The fusion protein was produced in E.coli strain JM101 and purified by amylose resin affinity chromatography. Mice were injected three times over six weeks with 200 μg of the purified protein. The first injection was in the presence of Freund's complete adjuvant, and the subsequent boosts in Freund's incomplete adjuvant. Two weeks after the final boost with a glutathione-Stransferase-cMOAT fusion protein, splenocytes were isolated and fused with myeloma cells. Hybridomas were screened by ELISA with the glutathione-S-transferase-cMOAT fusion protein and subsequently tested for positivity in Western blots.

cmoat cDNA was amplified from liver, kidney, ileum and duodenum from both Wistar and TR<sup>-</sup> rats using primers corresponding to amino acid residues 366-375 (forward) and 451-458 (reverse) of the cmoat sequence. The obtained PCR product was digested with NlaIII. In all PCR products from TR<sup>-</sup> rat digestion produced two bands of 206 and 66 bp whereas in the Wistar three bands of 83, 122 and 67 bp were observed.

Total RNA was extracted using the acid-phenol single step method [P. Chomczynski and N. Sacchi, Anal. Biochem. 8, 148 (1987)]. Poly(A)<sup>+</sup> RNA was isolated using the polyAtract mRNA system III (Promega, Madison). RNA was fractionated on a 0.8% denaturating agarose gel, transferred to Hybond N<sup>+</sup> nitrocellulose membrane filters and hybridized with a [a-

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32p]dCTP-labeled 213-bp rat lung mrpl probe and a 1-kb HindIII/AvaII fragment of cmoat in hybridization solution (11) for 20 hours at 65°C. Filters were washed 4x30 min in 0.2x SSC/0.1% SDS at 65°C and autoradiographed. A 32p-labeled 1.2-kb PstI fragment of the rat glyceraldehyde-3-phosphate dehydrogenase cDNA [Ph. Forth et al., Nucleic Acid Res. 13, 1431 (1985)] was used to estimate variations in RNA loading.

Canalicular and basolateral membranes were isolated as described by [P.J. Meier, E.S. Sztul, A. Reuben, J.L. 10 Boyer, J. Cell Biol., 98, 991 (1984)]. Membranes, containing 50  $\mu$ g of protein were fractionated by 7.5% SDS polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose filters, blocked for at least 2h in PBS/M/T (phosphate-buffered saline containing 1% BSA 15 and 1% milk powder and 0.05% Tween-20), and incubated with the monoclonal antibody ( $M_2$  III-5 hybridoma culture medium diluted eightfold with PBS/M/T) for 2h. Immunoreactivity was visualized with peroxidase-conjugated rabbit anti-mouse immunoglobulins and subsequent staining with 3,3'-20 diaminobenzidine and 4-chloro-1-naphthol substrate. Pglycoproteins were detected using the monoclonal antibody C219 and peroxidase-conjugated rabbit anti-mouse IgG. Immune complexes were visualized by enhanced chemiluminescence detection. 25

## Example 2. Isolation and characterization of the human cMOAT

The human homolog of the rat cMOAT cDNA was isolated using a 4 kb fragment of the rat cMOAT cDNA. The fragment 5 was labelled as described for the rat CMOAT cDNA. The labelled probe was then used to screen a human lambda gtll liver cDNA library. Three clones with inserts hybridizing with the rat cMOAT cDNA sequence were isolated and designated clone 12,7 and 20. Clone 12 contained an insert of 2716 nucleotides comprising coding sequence 130-2846. 10 Clone 7 contained an insert of 2000 nucleotides comprising coding sequence 2517-3185. Clone 20 contained an insert of 2231 nucleotides comprising the coding sequence 3069-5300. Missing nucleotides 1-130 encompassing the translation 15 initiation site were obtained from the WashU-Merck EST library, clone 1243479. Furthermore noncoding 3' sequences were found to be present in additional EST clones and were used to complete the full coding sequence of the human CMOAT cDNA. Clones 193244 and 199655 were used for this purpose 20 and completed the full length sequence from 5300 to 5582 nucleotides.

## Example 3. Transport experiments with rat cMOAT transfectants.

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The rat cMOAT cDNA was cloned into the mammalian expression vector pSVK3 (Pharmacia). pSVK3-rat-cMOAT and pSVK3 with rat-cMOAT in the reverse orientation (pSVK3-rat-cMOAT/Rev) relative to the promotor were transfected into COS-7 cells grown in 75 cm² tissue culture flasks. Three days after transfection, the cells were used for GS-DNP transport experiments and analyzed for cMOAT protein expression using anti-cMOAT antibodies. For transport measurements the cells were washed with Hanks buffer and loaded with Hanks/14C-CDNB at 15°C. Samples were taken after various time points. Input CDNB and cell mediated formation and transport of GS-DNP were separated by extraction of the samples with water-

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saturated ethylacetate. The water phase which contains the excreted  $^{14}\text{C}$  labelled GS-DNP was counted in a scintillation counter. Total protein was determined using the Lowry assay. GS-DNP efflux from the transfected cells was measured in a scintillation counter. Relative to COS-7 cells transfected with pSVK3-rat-cMOAT/Rev, cells transfected with pSVK3-rat-cMOAT excreted two fold more GS-DNP (See figure 5). This suggests that rat cMOAT transfected cells express a functional organic anion transporter protein in line with the expression of a protein reactive with anti-cMOAT antibodies. COS-7 cells transfected as described above were also used to isolate membrane vesicles and perform transport experiments. For this purpose cell homogenates were prepared from transfected COS-7 cells and were centrifuged over a 15 discontinous gradient of 19, 38 and 56 % sucrose. The 38-19 % interface was collected and revesiculated and total protein content was determined using the Lowry method. The vesicle suspensions were incubated wuth  $^3\mathrm{H}\text{-}\mathrm{GS}\text{-}\mathrm{DNP}$  at 37°C in the presence of an ATP regenerating system 20 After the indicated time points the vescile suspensions were filtered. Then the filters were washed with ice-cold stop buffer (250 mM sucrose, 20 mM HEPES/Tris pH = 7.4) and

counted in a scintillation counter. In agreement with the cell transport experiments, vesicles isolated from cells expressing rat-cMOAT exhibited GS-DNP transport above the level of transport observed with vesicles isolated from pSVK3-rat-cMOAT/Rev transfected COS-7 cells. This transport was completely dependent on the presence of ATP characteristic for a member of the ABC transporter superfamily (figure 6).

## Example 4: Transport experiments with human cMOAT transfectants

We have attempted to express the human cMOAT protein in several mammalian cell lines and in the yeast Saccharomyces

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sequences.

cerevisiae with various expression constructs carrying the human cMOAT cDNA. From 80 independant human cMOAT transfected and cloned human S1 cells only 2 clones expressed the human cMOAT protein at low levels. These expressing cells were tested for functional cMOAT by plating the cells in varying concentrations of doxorubicin, vincristin and cisplatinum.

The human LLC-PK1 cell line was also transfected with the same human cMOAT DNA construct and 90 clones were screened for expression. None of these clones expressed the human cMOAT protein as detected with antibody  $M_2III-6$ . In contrast, expression of human cMOAT in yeast was also studied and was high after the translational core sequences of the human cMOAT cDNA were converted to yeast consensus

However in both cases no active cMOAT mediated transport of GS-DNP could be observed. To investigate the role of cellular polarity in determining functional expression of the human cMOAT protein we have infected MDCK cells with an

- amphotropic retrovirus carrying the human cMOAT cDNA. For this purpose a HindIII-NcoI DNA fragment containing the complete predicted human cMOAT open reading frame was cloned into the retroviral vector pCMV-nec (Bender et al., 1987) resulting in a construct designated pCMV-neo-human-
- cMOAT. The retroviral amphotropic packaging cell line Phenix kindly provided by G.P. Nolan, Stanford University Medical Center, Stanford, USA) was cultured in Iscove's with 10% fetal calf serum. Phenix cells were transfected with pCMV-neo-human-cMOAT DNA using a commercially available calcium phosphate transfection kit (Gibco/BRL). After 16h at 5% CO2
  - , 37 °C, medium was changed and growth was continued for 48 h followed by collection of recombinant retrovirus containing medium. Storage of virus supernatants was at -20 °C. The MDCK cell line strain II (MDCKII; Louvard) was used
- for transduction experiments. For this purpose 2 x  $10^5$  MDCKII cells were seeded and incubated with a 5 ml 1/10 diluted virus stock in medium containing 30 mg Transfection

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Reagent (DOTAP; Boehringer Mannheim, Germany). After 10 h medium was replaced with fresh medium. Thirty six hours after infection cells were trypsinized and seeded at dilutions varying between 1/12-1/64. Stably infected cells were selected for 2-3 weeks in medium with G418 at 200 mg/ml. Thirty clones were picked and analyzed for the presence of hcMOAT protein. Western blot analysis of crude membrane fractions of these clones revealed that several clones contained a substantial, but between individual clones variable, amount of human cMOAT. Two of these clones 10 are shown in Figure 7. A weak signal was observed in wildtype MDCKII cells with a slightly higher molecular weight than hcMOAT after prolonged exposure. This might either represent canine cMOAT or another protein to which this mAb cross-reacts. The subcellular distribution of human cMOAT 15 protein was determined in MDCKII-217 hcMOAT transfectants, which showed the strongest signal in Western blot analysis (See figure 7). Cells were grown to confluency on microporous polycarbonate membrane filters (3 mm pore size,  $24.5 \text{ mm diameter, Transwell}^{\text{TM}}$  3414; Costar Corp., Cambridge, 20 MA) at a density of 2 x  $10^6$  cells per well as previously described (8). For confocal laser scanning microscopy, cells were washed in PBS and fixed for 10 min in aceton at rt. Filters were incubated with mAb  $M_2$ -III-6 (undiluted) for 60 min. Antibody binding was detected with a FITC-labeled sheep 25 anti-mouse IgG (1:50; Boehringer Mannheim, Germany). Filters were mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing propidiumiodide (1 mg/ml) for counterstaining of nucleic acids. Cells were examined with a MRC-600 confocal microscope (Bio Rad, Hertfordshire, UK). 30 Expression of hcMOAT protein was visualized by indirect immunolocalization using confocal laser scanning microscopy (CLSM). Clear staining was observed in approximately 50% of the cells (Figure 8 A), whereas in MDCKII wild-type only a very weak signal was detected (data not shown). Examination 35 of the cells at the plane perpendicular to the membrane filter revealed that almost all human cMOAT immunostaining

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was confined to the apical plasma membrane, although intracellular staining was observed in some cells (Figure 8 B).

To investigate whether human cMOAT expression in MDCK cells allows functional transport of GS-DNP the following experiment was performed. Export of  $[^{14}C]GS$ -DNP (GSH dinitrophenyl) from cells was determined by incubating cells with  $[^{14}C]CDNB$  ( $[^{14}C]$ -1-chloro-2,4-dinitrobenzene: 10 mCi/mmol) as described (8). The resulting hydrophilic GS-DNP only leaves the cell by active transport. Transport of GS-DNP across the apical and the basolateral membrane can be distinguished by growing cells as a monolayer on microporous membrane filters. Briefly, cells were grown on polycarbonate filters (see under immunocytochemistry) for 3-4 days. Two ml of medium (at room temperature) containing 2 mM [14C]CDNB was applied to both the apical and basal compartment of the monolayer and 200 ml aliquots were taken at various time points. After extraction with 200 ml of ethylacetate radioactivity in 160 ml of the water phase was determined by liquid scintillation counting. The amount of radioactivity was corrected for the decrease in volume of culture medium. To determine intracellular radioactivity, cells were washed with cold PBS, filters were cut from the plate and counted directly in liquid scintillation fluid. The resulting

pattern of GS-DNP export after exposing MDCKII, and the human cMOAT transfected clones is shown in Figure 9. MDCKII wild-type cells showed a substantial endogenous level of GS-DNP transport. Comparable transport to both the apical and basal compartment was measured in parental cells.

Remarkably, apical GS-DNP export was substantially higher in both human cMOAT transfected clones, demonstrating that human cMOAT is active as a glutathione conjugate pump in these cells (figure 9). Comparing the Western blot data (Figure 7) with transport data suggests that there is a correlation between the amount of human cMOAT detectable and

the level of apical GS-DNP transport. To exclude that differences in transport capacity between individual clones

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were due to differences in GST activity, and therefore differences in conjugation capacity, the total amount of GS-DNP retrieved in cells plus medium after 20 min was calculated. These data revealed that all clones had comparable levels of GS-DNP formed (data not shown).

Example 5: A mutation in the human cMOAT gene causes the Dubin-Johnson syndrome.

The human Dubin-Johnson syndrome is an autosomal recessive 10 liver disorder characterized by chronic conjugated hyperbilirubinemia. Patients have impaired hepatobiliary transport of non-bile salt organic anions a phenotype similar as has been described for the TR rat, which has a defective cmoat. In view of the identical phenotypes of TR-15 rats and Dubin-Johnson patients, we have tested whether a mutation in the human cMOAT gene also underlies the transport defect in Dubin-Johnson syndrome. In this example we demonstrate that the human homologue of rat cMOAT, human CMOAT also subject of this invention, is deficient in a 20 patient with Dubin-Johnson syndrome. Furthermore we show that we have used the DNA sequence of the human  $c\emph{MOAT}$  cDNA to develop two diagnostic assays for Dubin Johnson syndrome.

We have studied a female, caucasian patient (age 54) who was diagnosed for Dubin-Johnson at the age of 20. She 25 frequently complained of pains in the upper abdomen. General liver function was normal except for elevated conjugated (38 to 70  $\mu\text{M}$ ) and unconjugated (12 to 25  $\mu\text{M}$ ) serum bilirubin levels. It was not possible to visualize the gallbladder after administration of oral contrast reagent, a 30 characteristic feature of Dubin-Johnson. The patient demonstrated a delayed plasma clearance of i.v. injected BSP, followed by a secondary rise in plasma BSP levels. At the age of 32 the patient underwent cholecystectomy. A characteristic black liver was observed and microscopic 35 analysis of a liver section revealed mild fibrosis and the pigment accumulation indicative of Dubin-Johnson. Liver from

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this patient was obtained by a needle biopsy. Normal control liver was obtained from surgical pathology specimens. Biopsies were fixed for histology in 4 % formaldehyde and embedded in paraffin. Skin fibroblasts from the patient and a normal control were obtained by skin biopsy and cultered in Ham F-10 (Life Technologies), supplemented with 10 % fetal bovine serum and antibiotics, at 37  $^{\circ}$ C.

Paraffin-embedded liver sections of Dubin-Johnson and control liver were examined for the presence and localization of the cMOAT protein, using monoclonal antibody 10 M2III-6. For this purpose formaldehyde-fixed paraffinembedded liver sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 0.3 % (v/v) H<sub>2</sub>O<sub>2</sub> in methanol for 30 min. Before staining, the sections were pretreated with 0.01 M citric acid (pH 15 6.0) for 3 x 5 min at 100°C. The sections were blocked with normal rabbit serum for 10 min and incubated with monoclonal antibody M2III-6 for 1 h. Immunoreactivity was visualized with biotinylated rabbit anti-mouse Fab2 (Dako Copenhagen, Denmark), followed by streptavidin-conjugated horseradish 20 peroxidase (Dako) in PBS/1% BSA, and subsequent staining with 3,3'-diaminobenzidine tetrahydrochloride and 0.02 % (v/v)  $H_2O_2$  in PBS. P-glycoproteins were detected with monoclonal antibody JSB-1. All sections were (counter) stained with hematoxylin and mounted. The  $M_2III-6$ 25 antibody was produced against a bacterial fusion protein containing the 202-amino acid COOH-terminus of rat cMOAT; it cross-reacts with human cMOAT, but not with human MRP1. In human control liver, like in rat control liver, the antibody stained the canalicular membrane of the hepatocyte. In 30 Dubin-Johnson liver , as in TR rat liver, no canalicular staining was observed, indicating that this patient lacks the cMOAT protein (figure 11). A positive canalicular staining was observed in both Dubin Johnson syndrome and control liver with JSB-1, an antibody against P-35 glycoprotein, used as a positive control. To investigate the

nature of the genetic defect, total RNA was isolated from

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cultured fibroblasts obtained from a skin biopsy of both the patient and a normal control according to the acid-phenol single step method. cDNA synthesis was carried out with 6  $\mu g$ of total RNA and random hexamer primers with Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV-RT, Life Technologies, Gaithersburg, MD), at 37°C for 1 h, followed by 10 min 65°C to inactivate the M-MLV-RT. The complete CMOAT cDNA was amplified by the "touch down" PCR protocol from both patient and control fibroblast cDNA using five sets of cMOAT-specific primers: 5'-TAGAAGAGTCTTCGTTCCAGACGCAG-3'(forwardI) and 5'-GCAATTTCAGCAGCTGAGGACTCAC-3'(reverseI), 5'-AAATCCTGGTTGATGAAGGCTCTG-3'(forwardII) and 5'-TCCAGGTTCACATCTCGGACTCTGGC-3'(reverseII), 5-ACATCTGCCATTCGAGATGACTGC-3'(forwardIII) and 5'-CAACTCTCATGTCCCTCTGAGATGC-3'(reverseIII), 5'-TGAAGTTCTCCATCTACCTGGAGTACC-3'(forwardIV) and 5'-GATGATGGTCAGCTTCTCTCGGAGG-3'(reverseIV), and 5'-GTCATCCCTCACAAACTGCCTCTTCAGAATCTTAG-3'(forwardV) and 5'-CTGCTAGAATTTTGTGCTGTTCACATTC-3'(reverseV). PCR reactions were carried out in a Perkin Elmer GeneAmp PCR system 2400, in 1x Tag polymerase buffer (Life Technologies), 1.5 mM of  $MgCl_2$ , 0.5 mM of dNTPs, 400 nM of each primer, and 0.5 units of Taq polymerase. The PCR products were obtained after application of the "touch down" PCR protocol; the reactions were denatured at 96°C for 5 min, followed by five times 2 cycles with annealing temperatures of 72, 70, 68, 66, and 65°C respectively, and subsequent 30 cycles with an annealing temperature of 64°C. Each cycle started with 20 s at 94°C, 30s at the indicated annealing temperature, and 90s at 72°C. The PCR reaction was terminated after an extension step at 72°C for 10 min. PCR fragments obtained from fibroblasts were excised from agarose gel, purified, ligated into the TA-cloning plasmid  $pCR^{TM}II$  (Invitrogen, Leek, The Netherlands), and transformed into INVaF' competent cells (Invitrogen). White colonies were picked, grown overnight,

and plasmid DNA was isolated using the alkaline lysis

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method. Nucleotide sequences of 5-8 pooled clones were determined by the dideoxynucleotide chain method. Sequence analysis of multiple independent clones revealed a mutation in the patient at codon 1066 (CGA to TGA; arginine to stopcodon) (figure 12), which leads to premature termination of cMOAT protein synthesis, the normal protein being 1545 amino acids long (see also figure 10). The mutation results in the loss of a TaqI restriction site, and we have confirmed the absence of this site in the patient by TaqI digestion of her cMOAT cDNA (figure 13). From this observation we conclude that this patient is either homozygous for the mutation in codon 1066, or that the second allele does not give rise to a mRNA.

In conclusion, the identification of a mutation in human

cMOAT in a patient with the DJS confirms the hypothesis that
the TR<sup>-</sup> rat is an animal model for Dubin Johnson syndrome
and provides additional evidence that the cMOAT gene encodes
the major transporter for organic anions in the liver
canalicular membrane. Our demonstration of a low, but
detectable expression of the cMOAT gene in fibroblasts in
addition to a nucleic acid based diagnostic assay for DubinJohnson syndrome, allows a simple identification of this
inherited disorder, without the need for liver biopsy.

25 Example 6: Identification and isolation of other members of the family of anorganic anion transporters.

#### 6.1 Materials and Methods

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#### 6.1.1 Cell lines

All cell lines used in this study have been described in the literature before: the drug-sensitive and doxorubicin-selected MDR sublines of the non-small-cell lung cancer cell lines SW1573/S1 and COR-L23 (58-61); the small cell lung cancer cell line GLC4 (62); the lung adeno carcinoma cell line MOR/P (61); and the laukemia cancer cell line HL60

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(63); the T24 bladder carcinoma cell line and three CDDPresistant sublines (45); the 2008 ovarium carcinoma cell line, two CDDP-resistant sublines and a  $Cd^{2+}$ -resistant subline (40, 41); the A2780 ovarium carcinoma and the HCT8 colon carcinoma cell lines and CDDP-resistant sublines of both (39, 47); the PXN94 ovarium carcinoma and the tetraplatin-resistant subline PXN94tetR (42); the GCT27 testicular carcinoma cell line and the CDDP-resistant subline GCT27cisR (44); the KB-3-1 epidermoid carcinoma cell line and a CDDP-resistant subline KCP-4 (37, 38). All cells 10 were grown in DMEM or RPMI medium (Gibco, BRL), supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (50 units/ml) and streptomycin (50  $\mu$ g/ml). All cells were free of Mycoplasma as tested by the use of the Gene-Probe rapid Mycoplasma detection system (Gen-Probe, San Diego, USA). 15

## 6.1.2 Clonogenic survival assays

The drug sensitivity of cells was determined in clonogenic survival assays in the continuous presence of drugs. Five hundred cells per well were seeded in 24-well plates and incubated for 24 hrs at 37°C. Drugs, of which concentrations were varied in 2-fold steps, were added and cells were incubated for 5-6 days at 37°C. After this the cells were stained with 0.2% crystal violet in 3.7% glutaraldehyde and colonies containing more then 50 cells were counted. The relative resistance was calculated as the ratio of IC50 (Inhibitory Concentration where 50% of the cells survives) of the resistant cell line to the IC50 of the parental cell line.

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# 6.1.3 Cloning and sequencing of MRP3, MRP4, and MRP5

For the isolation of MRP3, MRP4, and MRP5 cDNA, human cDNA clones were obtained from the I.M.A.G.E. consortium (64). Additional MRP3 cDNA clones were isolated by screening a human liver 5' stretch plus cDNA library, oligo(dT) and random primed (Clontech, Palo Alto, USA), using a 1 kb

EcoRI-SacI fragment of a human cDNA clone (no. 84966, Stratagene liver cDNA library #937224) as probe. Several overlapping cDNAs were isolated and sequenced. For MRP4 the insert of a human cDNA clone (no. 38089, Soares infant brain 1NIB cDNA library) was sequenced, containing the 3'-terminal 5 end of the gene. MRP5 cDNA clones were isolated by screening a fetal brain cDNA library (Clontech, Palo Alto, USA), using the insert of human cDNA clone (no. 50857, Soares infant brain 1NIB cDNA library) as probe (J. Wijnholds, C. Mol, and P. B., unpublished results). Several overlapping cDNAs were 10 isolated and sequenced. For sequencing the ABI 377 Automatic Sequencer was used. Sequence analysis was done using the GCC package of the Wisconsin University (20). All the sequences have been deposited with GenBank (MRP3 accession number U83659; MRP4 accession number U83660; MRP5 accession number 15 U83661).

#### 6.1.4 RNA

Cytoplasmic RNA from cell lines was isolated by a Nonidet P40 lysis procedure (24). Total cellular RNA from tissue samples obtained during surgery or at autopsy was isolated by acid guanidium isothiocyanate-phenol-chloroform extraction (65).

### 25 6.1.5 RNase protections

By PCR amplification of human *cMOAT* cDNA a 241 bp fragment corresponding to nucleotides (nts) 4136-4376 (49; GenBank accession number U49248) was generated. The primers used for amplification were 5'-CTGCCTCTTCAGAATCTTAG-3' (forward

- primer) and 5'-CCCAAGTTGCAGGCTGGCC-3' (reverse primer). For MRP3, MRP4, and MRP5 RNA detection the following fragments were generated by PCR amplification: (i) for MRP3 a 262 bp fragment was generated using the primers
  5'-GATACGCTCGCCACAGTCC-3' (forward primer) and
- 5'-CAGTTGGCCGTGATGTGGCTG-3' (reverse primer); (ii) for MRP4 a 239 bp fragment was generated using the primers 5'-CCATTGAAGATCTTCCTGG-3' (forward primer) and 5'-

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GGTGTTCAATCTGTGTGC-3' (reverse primer); (iii) for MRP5 a 381 bp fragment was generated using the primers 5'-GGATAACTTCTCAGTGGG-3' (forward primer) and 5'-GGAATGCCAATGCTCTAAAG-3' (reverse primer). All the fragments were cloned into pGEM-T (Promega, Madison, USA), 5 resulting in the plasmids hcMOAT-241, MRP3-262, MRP4-239, and MRP5-381, and the sequences were confirmed. For RNase protections,  $a^{-32}p$ -labeled RNA transcripts were transcribed from NotI-linearized DNA of hcMOAT-241 and MRP3-262, using T7 RNA polymerase, or from NcoI-linearized DNA from MRP4-239 10 and MRP5-381, using Sp6 RNA polymerase. For MDR1 RNA detection, a 301 bp MDR1 cDNA fragment was used (nt positions 3500-3801 (66)), and for MRP1 RNA detection a 244 bp MRP1 cDNA fragment was used (nt positions 239-483 (7)). RNase protections were carried out according to Zinn et al. 15 (67), modified by Baas et al. (58). Protected probes were visualized by electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. In all experiments a probe for actin (68) was included as control for RNA input. The amount of MDR1, MRP1, cMOAT, MRP3, MRP4, 20 or MRP5 RNA relative to the amount of actin was calculated using a phosphorimager (Fuji BAS 2000, TINA 2.08b).

## 6.1.6 Protein analysis

Total cell lysates were made by lysing harvested cells in 10 25 mM KCl/1.5 mM MgCl<sub>2</sub>/10mM Tris-HCl, pH 7.4/0.5% (wt/vol) SDS supplemented with 1 mM phenylmethylsulfonyl fluoride, leupeptin (2  $\mu$ g/ml), pepstatin (1  $\mu$ g/ml), and aprotinin (2  $\mu$ g/ml). DNA was sheared by sonication and samples containing 40  $\mu g$  of protein were fractionated by SDS/7.5% PAGE and then 30 transferred onto a nitrocellulose filter by electroblotting. After blotting the filters were blocked for at least 2 hours in Blotto (Phosphate-buffered saline containing 1% bovine serum albumin, 1% milk powder, and 0.05% Tween-20), followed by incubation for 2 hours with the primary antibody in 35 Blotto. cMOAT protein was detected with mouse monoclonal antibodies  $M_2III-5$  or  $M_2III-6$ , generated against a bacterial

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fusion protein containing the 202 amino acid COOH-terminus of rat cmoat (48). Immunoreactivity was visualized with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Denmark) followed by enhanced chemiluminescence detection (Amersham, U.K.).

- 6.1.7 Fusion proteins of cMOAT, MRP3, and MRP5.

  To test the cross-reactivity of the cmoat monoclonal antibodies with human cMOAT and other MRP homologs, fusion proteins were made of the Escherichia coli maltose-binding protein with COOH-terminal ends of human cMOAT, MRP3, and MRP5, respectively, using the plasmid vector pMal-c (69). The expression plasmids encoded, respectively, for cMOAT the 202-amino acid COOH-terminal end, for MRP3 the 190-amino acid COOH-terminal end, and for MRP5 the 169-amino acid COOH-terminal end. The fusion proteins were produced in E.coli DH5a and purified by amylose resin affinity chromatography (69).
- 20 6.1.8 Glutathione assay
  Cells (1-2 x10<sup>6</sup> per well) were plated in triplicate in 6
  wells plates in medium with or without drugs. 48 hrs after
  plating the cells were washed with phosphate-buffered saline
  and scraped in 10% perchloric acid. Precipitated protein was
  removed by centrifugation and the supernatant was
  neutralized by adding 0.5 M MOPS/5 M KOH. The concentration
  of total glutathione (GSH and glutathione disulfide (GSSG))
  was determined according to the recycling method of Tietze
  (70).

6.1.9 Chromosome localizations

For the chromosome localization of MRP3, MRP4, and MRP5, radiation hybrid mapping was performed with MRP3, MRP4, and MRP5 specific primers and two different cell panels, Stanford G3 (StG3; 71) and Genebridge 4RH (Gb4RH; 72). The primers used for amplification were: (i) for MRP3

5'-CTCAATGTGGCAGACATCGG-3' and 5'-GGGAGCTCACAAACGTGTGC-3';

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(ii) for MRP4 5'-CCATTGAAGATCTTCCTGG-3' and 5'-GGTGTTCAATCTGTGTGC-3'; (iii) for MRP5 5'-CCTGTTTGGGAAGGAATATGA-3' and 5'-GGGTCGTCCAGGATGTAGAT-3'. For the PCR reactions 25 ng DNA, 2 ng/ $\mu$ l of each specific primer, 0.8 units Goldstar polymerase (Eurogentec, Seraing, 5 Belgium) (MRP3 and MRP4) or 1.5 units Amplitaq Gold polymerase (MRP5) were used in a total volume of 25  $\mu$ l with 1.5 mM MgCl  $_2$  and 100  $\mu M$  of each dNTP at final concentrations. The PCR conditions were: initial denaturation 5 min 94°C (MRP3 and MRP4) or 12 min 95°C 10 (MRP5), followed by 42 cycles of 15 sec 94°C, 30 sec 58°C, and 45 sec 72°C. Final extension was for 10 min at 72°C. PCR products were resolved by 1 percent agarose gel electrophoresis and the cell line scored positive, negative or ambiguous for presence of the gene. Datafiles were 15 submitted to the Stanford Human Genome Center or Whitehead Institute radiation hybrid mapping databases for placing of the MRP genes in context of the respective radiation hybrid map framework markers.

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## 6.1.10 Microsatellite repeat analysis

To confirm identity of cell lines and subclones 9 highly polymorphic microsatellite markers were used (D1S1649, D2S434, D2S1384, D3S2427, D9S301, D9S934, D12S2070, D14S611, and D17S969). PCR conditions were as described in the Genome Database (GDB). One primer of each set was labelled with a fluorescent dye and PCR products were visualized by electrophoresis on a ABI 377 automatic sequencer. Data were analyzed with Genetyper software version 1.1.1 (Perkin Elmer, Foster City). Allele sizes were within expected range.

### 6.1.11 deposited clones

The sequences for human MRP-3, MRP-4, MRP-5 and for human and rat cMOAT are deposited at ECACC (European Collection of Cell Cultures, Salisbury, Wiltshire SP4 OJG, UK) under provisional numbers.

96010801-hu-MRP3 #96A 96010802-hu-MRP5 #97 96010803-hu-MRP4 #38089 96010804-hu-cMOAT #33A 5 96010805-hu-MRP3 #20.11 96010806-hu-MRP5 #101 96010807-hu-MRP5 #104 96010808-hu-MRP5 #105 96010809-hu-MRP3 #20.1 10 96010810-rat-cMOAT 96010811-hu-MRP3 #97F

#### 6.2 Results

We searched human EST databases (dbEST, TIGR) for MRP homologs other than MRP1 and cMOAT. Alignment and comparison of EST sequences with homology specific to the 3'-terminal ends of MRP1 and cMOAT, including the coding sequence for the second ATP-binding domain, revealed that there are at least 4 more MRP homologs expressed in humans. One of these homologs is the human sulfonylurea receptor (SUR) gene (73). The other three MRP homologs had not been identified before, and were designated MRP3, MRP4, and MRP5.

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## 6.2.2 Cloning and sequencing of MRP3, MRP4, and MRP5 cDNA

Additional cDNA clones for MRP3 and MRP5 were isolated from a human liver and a fetal brain cDNA library, respectively. MRP3 and MRP5 cDNA clones were sequenced as well as the MRP4 cDNA clone obtained from the I.M.A.G.E. consortium. Both MRP3 and MRP5 encode four domain proteins, i.e. proteins with two ATP-binding domains and two domains with transmembrane regions (M.K. and J. Wijnholds, unpublished results). More sequence data will determine whether this is also the case for MRP4. Figure 14 shows the protein alignment for the COOH-terminal ends of the various members

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of the human MRP family and human SUR. The alignment includes the Walker A and B motifs and the signature sequence of the second ATP-binding domain. The percentages of homology for the COOH-terminal 124 amino acids are shown in table 1. The highest homology is found between MRP1 and MRP3 (86% similarity) and the lowest between SUR and any of the MRPs ( $\leq$  69% similarity).

6.2.3 Chromosome localization of MRP3, MRP4, and MRP5 The MRP1 gene has been mapped to chromosome 16 at band 10 pl3.13-13.12 (5) and recently the cMOAT gene to chromosome 10, band q24 (52, 74). We mapped the other MRP homologs on the Gb4RH and StG3 radiation hybrid mapping panels, using MRP3, MRP4, or MRP5 specific primers. MRP3, MRP4, and MRP5, are located on chromosomes 17, 13, and 3, respectively. The 15 most closely linked markers were D17S797 (Gb4RH) and D17S1989 (StG3) for MRP3, WI-9265 (Gb4RH) and D13S281 (StG3) for MRP4, and WI-6365 (Gb4RH) and D3S4205 (StG3) for MRP5. These results are consistent between the radiation hybrid mapping panels and demonstrate that the new MRP homologs are 20 indeed new genes, and not splice variants of MRP1 or cMOAT.

# 6.2.4 Human tissue distribution of cMOAT, MRP3, MRP4, and MRP5 RNA

25 RNase protections were performed to determine the tissues that express cMOAT and MRP3, MRP4, and MRP5. The results are summarized in table 2. Both cMOAT and MRP3 are highly expressed in liver, and to a lower extent also in duodenum. Low expression of cMOAT was found in kidney and peripheral nerve. For MRP3, substantial expression, similar to expression in duodenum, was also detected in colon and adrenal gland. MRP4 is expressed at a low level in only a few tissues tested. MRP5 RNA was detected in substantial amounts in every tissue tested, with relatively high expression in skeletal muscle and brain.

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6.2.5 Expression of MRP homologs in resistant cell lines

In view of their homology with MRP1, cMOAT and the three new MRP homologs are believed to encode transporter proteins

involved in drug resistance. We therefore screened a large set of human cell lines derived from various tissues and their resistant sublines selected with either doxorubicin, cisplatin, tetraplatin, or CdCl<sub>2</sub>. Only resistant lines showing decreased cellular accumulation of drugs were

analyzed. All cell lines were analyzed by RNase protection for levels of MDR1, MRP1, cMOAT, MRP3, MRP4, MRP5, and actin RNA. The results are summarized in tables 3 and 4, and an example of each probe is shown in Fig. 15.

High MDR1 overexpression was detected only in two 15 sublines of the human non-small-cell lung cancer cell line SW1573/S1, both selected for high level doxorubicin resistance (2R160 and 1R500). The low level of MDR1 RNA in the other cell lines is not remarkable as most of the cell lines selected for our panel were known to have a non-Pgp 20 MDR phenotype. Low MDR1 overexpression was found in the 2R120, a subline of the SW1573/S1, and in three cisplatin selected sublines of the bladder carcinoma cell line T24. Interestingly, a decrease rather than an increase in MDR1 RNA was seen in two cisplatin selected sublines of the 25 ovarium carcinoma cell line 2008 (table 4). This phenomenon has been reported earlier in the SW1573/S1 sublines 1R50b, 2R50, and 3R80, selected for low level doxorubicin resistance (58, 59; Table 3).

MRP1 RNA is highly overexpressed in the four non-Pgp
30 MDR cell lines GLC<sub>4</sub>/ADR, MOR/R, COR-L23/R, and HL60/ADR, all
selected for high level doxorubicin resistance (7, 75, 76).
The doxorubicin selected cell lines, derived from the
SW1573/S1 cell line, showed no or only a minor increase in
MRP1 RNA, as reported before (7, 77). In the cell lines,
selected for cisplatin resistance, we detected no major
changes in MRP1 RNA. Only in two sublines of the T24 cell
line, T24/DDP7 and T24/DDP10, and in HCT8/DDP, a subline of

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the colon carcinoma HCT8 cell line, a slight (less than 2 fold) increase in MRP1 RNA was found.

Expression of cMOAT varied greatly between the cell lines. Most parental cell lines did not express cMOAT or at very low levels. Only the MOR/P and the KB-3-1 parental cell lines showed substantial cMOAT RNA levels. Overexpression of cMOAT was found in several doxorubicin-resistant sublines of SW1573/S1 (30.3M, 1R50b, 2R120, 2R160, and 1R500), and some cisplatin selected cell lines (2008/C13\*5.25, 2008/A,

10 A2780/DDP, and HCT8/DDP).

Similar to *cMOAT*, most parental cell lines either did not express *MRP3* or only at very low levels. The only two parental cell lines, which show high expression of *MRP3*, the MOR/P and the KB-3-1, also show high expression of *cMOAT*. Overexpression of *MRP3* in resistant lines was only found in several doxorubicin-resistant sublines of the SW1573/S1 cell line and the cisplatin resistant HCT8/DDP cell line.

 $\it MRP4$  is expressed only at low or very low levels in the cell lines we analyzed and no overexpression of  $\it MRP4$  was detected in resistant sublines.

MRP5 is expressed in every cell line we analyzed, with the highest levels in MOR/P and 2008, but in none of the resistant sublines MRP5 is highly overexpressed. Only in three cisplatin resistant cell lines, T24/DDP10, HCT8/DDP, and in the KCP-4(-), a minor increase in MRP5 RNA was detected.

6.2.6 cMOAT protein in resistant cell lines
To investigate whether the increased cMOAT RNA levels in the resistant cell lines were accompanied by increased cMOAT protein levels, total cell lysates were tested on Western blot with the monoclonal antibodies M2III-5 and M2III-6, generated against amino acids 1340 to 1541 of the rat cmoat protein (48). To test the specificity for human proteins of the Mabs generated against rat cmoat, fusion proteins containing COOH-terminal ends of human cMOAT, MRP3, and MRP5, were made. Both cMOAT Mabs, M2III-5 and M2III-6,

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recognize human cMOAT.  $M_2$ III-5 also reacts with the MRP5 fusion protein, and  $M_2$ III-6 also reacts with the MRP3 fusion protein. No cross-reaction was detected for both Mabs with MRP1 (data not shown).

Protein analysis of the cell lines with the cMOAT Mabs showed the presence of a 190-200 kDa protein in several lines (Fig. 16). Similar results were obtained with M2III-5 and with M2III-6 (not shown), indicating that the protein detected is cMOAT. The level of cMOAT protein in each cell line correlated very well with the level of cMOAT RNA, even for the cell lines with only a marginal increase in cMOAT RNA, such as the 2008/Cl3\*5.25 and the 2008/A. The only exception was the cisplatin resistant subline of KB-3-1. KCP-4(-). The Western blot shows that the cMOAT protein level was about 2-3 fold higher in the KCP-4(-) cell line than in the KB-3-1, whereas the RNA levels were the same in parental and resistant cells. Mab M2III-5 also reacts with MRP5 and MRP5 RNA is raised in the KCP-4(-) cells, but a similar result was obtained with Mab M2III-6 which does not

All cell lines with no or only very low levels of CMOAT RNA also contained no detectable cMOAT protein (Fig. 16). The small amount of cMOAT detected in the parental A2780 cell line migrated faster in the gel than the cMOAT protein present in the cisplatin resistant A2780/DDP cell line, or the protein detected in the HCT8, HCT8/DDP, KB-3-1, and KCP-4(-) cells. The varying mobility of cMOAT in the gel could be caused by different degrees of post-translational modification of cMOAT protein in each cell line, as we have observed for MRP1 (77; M.K. unpublished results), but this needs to be verified.

# 6.2.7 Glutathione assays

cross-react with MRP5.

In view of the proposed role of cMOAT as a GS-X pump,

intracellular GSH levels were measured for the cell lines in table 4. GSH levels were elevated in all resistant cell

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lines (Table 4), and were not detectably different in cells cultured with or without drugs (data not shown).

- 6.2.8 Drug resistance of the cell lines analyzed
  To determine whether there is a correlation between the
  elevation of expression of transporters and resistance
  pattern, we have extended the existing information on these
  cell lines with a more complete survey of resistance against
  either cisplatin or doxorubicin (Table 5). Interestingly,
- all the doxorubicin selected SW1573 cell lines with overexpression of cMOAT are also cross-resistant against cisplatin, and the level of cMOAT expression correlates quite well with the level of cisplatin resistance (Tables 3 and 5). Cytotoxicity analysis of the KCP-4(-) cell line
- showed that the IC<sub>50</sub> for cisplatin for this cell line was much lower than reported (700 nM, RF 1.8 [table 5] instead of 25.000 nM, RF 62.5 [37]), suggesting that this cell line was a revertant or contaminated with another low-level-cisplatin resistant cell line. When these KCP-4(-) cells
- were cultured in the presence of 6.7 µM cisplatin, more than 99% of the cells died. The surviving population, KCP-4(+), was highly cisplatin resistant again (IC50 22.400, RF 59 [table 5]), but did not express CMOAT anymore (Fig. 16). Microsatellite repeat analysis showed that both cell lines,
- 25 KCP-4(-) and KCP-4(+), were derived from the parental KB-3-1, indicating that the KCP-4(-) is most likely a revertant.

All cell lines selected for resistance against cisplatin, tetraplatin or  $CdCl_2$  are not cross-resistant against doxorubicin (Table 5), with two exceptions: the KCP-4(-) cell line and the PXN94/tetR cell line. Cross-resistance did not correlate with *cMOAT* expression.

# 6.3. Discussion.

35 **6.3.1.The MRP gene family**Our database search of expressed sequence tags has revealed that at least five homologs of MRP1 are expressed in man.

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cMOAT or MRP2 encodes the major organic anion transporter in the canalicular membrane of hepatocytes (48-52, 55). The product of another homolog, SUR, plays a role in the regulation of insulin secretion (73). The other three homologs, MRP3-5, are all more related to MRP1 than SUR (Table 1). Identity is highest between MRP1 and MRP3 (75%). Since the region taken for comparison is small and one of the most conserved parts of the protein, the overall identity between the MRP homologs will probably be lower than the percentages in Table 1.

The MRP homologs MRP3-5 are all located on other chromosomes than MRP1 and cMOAT. This confirms that MRP3, MRP4, and MRP5 are not alternative splice products of MRP1 or cMOAT. Klugbauer and Hofmann (78) recently cloned another 15 ABC transporter (ABC-C), located in the same chromosomal band as MRP1, but this is not a MRP homolog, because the identity between these two proteins is only 18%. After our work was completed Allikmets et al. (79) reported the identification of 21 new ABC genes also based on a search of the human EST database and they mapped the identified partial sequences.

# 6.3.2 Physiological functions of the MRP family members

The physiological role of these new MRP proteins is probably a role in cellular detoxification processes by exporting GSH S-conjugates or other organic anions. GSH S-conjugate 5 carriers have been described in many mammalian cells, including liver, heart, lung, and mast cells and erythrocytes (1B, 80). Kinetic studies indicate that both liver canaliculi and erythrocytes contain two different ATPdependent transport activities for organic anions (81-84). 10 cMOAT is localized in the canalicular membranes of hepatocytes and the absence of this protein in the TR- rats as well as in a patient with the Dubin-Johnson syndrome shows a role for the cmoat/cMOAT proteins in the transport of non-bile acidic organic compounds from liver to bile (48, 15 49, 55). The other ATP-dependent transport activity in liver canaliculi, responsible for transport of bile acids from liver to bile is not attributable to cMOAT, because studies with TR rats and Dubin-Johnson patients showed that bile acid transport was not affected (1B). 20

Two other congenital liver diseases characterized by a conjugated hyperbilirubinemia, like the Dubin-Johnson syndrome, are Benign Recurrent Intrahepatic Cholestasis (BRIC) and Progressive Familial Intrahepatic Cholestasis (PFIC or Byler disease) (85, 86). The clinical and biochemical features of BRIC and PFIC are suggestive of a defect in primary bile acid secretion (87, 88). BRIC and PFIC have both been mapped to the same region on chromosome 18, 18q21-q22 (89, 90).

In view of the high expression of MRP3 in the liver (Table 2), MRP3 may be the bile salt transporter. Since none of the human ABC transporter genes identified thus far maps to chromosome 18 (79; this study) it is unlikely that BRIC/PFIC is caused by a defect in a readily recognizable ABC transporter gene.

GS-X activity has also been found in erythrocytes. Several studies have shown that human and rat erythrocytes

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contain a low- and a high-affinity S-(2,4-dinitrophenyl)-glutathione (DNP-SG) transporter (84, 91, 92). The high-affinity DNP-SG transporter is most likely MRP1, since the presence of this protein and its binding to LTC4 have been shown for erythrocytes (93, 94). The other transporter with low affinity for DNP-SG but high affinity for glucuronides and mercapturates (84) is not cMOAT or the bile salt transporter, because (i) no major alterations in DNP-SG transport in erythrocytes from TR<sup>-</sup> rats and Dubin-Johnson patients were detected (1B), and (ii) erythrocytes transport DNP-SG and GSSG but no bile salts (83). This second transporter may be encoded by one of the other MRP homologs.

# 6.3.3 Expression of MRP homologs in resistant cell lines

We screened a large set of cell lines and their resistant sublines to see whether MRP1, cMOAT or one of the other MRP homologs is overexpressed. MRP4 was not overexpressed in any of the lines. MRP3 RNA was only found to be elevated in the cisplatin resistant HCT8/DDP cell line and several SW1573/S1 sublines selected for doxorubicin resistance. However, overexpression did not correlate with the level of doxorubicin resistance. For MRP5 low overexpression was found in three cell lines selected for cisplatin resistance (T24/DDP10, HCT8/DDP, and KCP-4(-); Table 4), but many other cisplatin selected cell lines showed no overexpression.

Table 3 shows that the classical non-Pgp cell lines selected for high doxorubicin resistance and known to highly overexpress the MRP1 gene, do not significantly overexpress other members of the MRP family. This is compatible with the interpretation that MRP1 is the transporter responsible for MDR in these cell lines. In the non-Pgp derivatives of the SW1573/S1 cell line presented in Table 3 a more complex situation is found and the contribution of MRP1, cMOAT, MRP3, and the major vault protein, also present at increased levels in some of these cell lines (95), remains to be sorted out.

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6.3.4 The involvement of organic anion transporters in cisplatin resistance

Whereas P-glycoproteins do not transport small or highly
charged molecules, organic anion transporters, such as MRP1
and cMOAT have been speculatively linked to resistance to
oxyanions (arsenite, antimonite) and cisplatin. These
compounds can form complexes with GSH and there is now
considerable evidence that these complexes are substrates
for organic anion transporters. Resistance caused by
increased export of these complexes is bound to be complex,
as pointed out by Ishikawa (80) and by us (6b, 19, 95).
Increased levels of pump or GSH, increased GSH synthesis, or
a combination may be required depending on the rate limiting
step in drug export.

In the protozoal parasite Leishmania, resistance to arsenite and antimonite can be associated with both a 40fold increase in the Leishmania GSH homolog trypanothione (97) and an increase in the MRP-related ABC-transporter PgpA (98). Cancer cells selected for high levels of cisplatin may sometimes also contain extremely high concentrations of GSH (99) and the GSH synthesis in these cells is upregulated (99-101). All of the cisplatin resistant cell lines studied by us have elevated GSH levels as well, albeit not as high as the cell lines isolated by Godwin et al. (99). In contrast to published data, we also find raised GSH levels in the T24 sublines, the GCT27cisR, and the PXN94/tetR cell lines (42, 44, 45). We find no clear correlation, however, between the degree of cisplatin resistance and GSH levels, as observed by Godwin et al. (99). Moreover, all the cell lines studied by us show a decreased accumulation of cisplatin and an organic ion pump may therefore be involved in resistance.

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Ishikawa et al. (36) showed that MRP1 is overexpressed in the cisplatin resistant human leukemia cell line HL60/R-CP. They concluded that an increased GSH synthesis in combination with raised MRP1 levels can cause cisplatin

resistance. Active cisplatin efflux has been described in three of the cell lines in Table 4: KCP-4, A2780/DDP, and HCT8/DDP (37-39, 102). The ATP-dependent efflux was inhibited by DNP-SG, indicating that it was catalyzed by a 5 GS-X pump. In addition, the membrane vesicles of the KCP-4 cell line were shown to catalyze an increased uptake of  $LTC_4$ (37, 38), known to be the substrate with the nighest affinity for MRP1. However, data from these papers and our study show that MRP1 is not overexpressed in these cisplatin resistant cell lines, suggesting that MRP1 is not the major 10 pump responsible for cisplatin resistance. This is supported by transfection studies with MRP1, which showed no cisplating resistance of the transfected cells (28, 17). Nevertheless, it remains possible that transport of cisplatin conjugates by MRP1 is efficient and that the low levels of MRP1 present 15 in parental cells suffice for resistance, if formation of cisplatin conjugates in resistant cells is increased, e.g. by an increase in GSH synthesis.

An organic anion pump that is important in cisplatin 20 resistance is cMOAT. Especially striking is the correlation between cisplatin resistance and cMOAT expression in the non-Pgp MDR cell lines derived from the SW1573/S1 cell line (Table 5). These lines were selected for doxorubicin resistance and it is therefore unlikely that other 25 mechanisms of cisplatin resistance are activated in these lines. It should be noted, that these non-Pgp MDR lines, selected for low level doxorubicin resistance, contain multiple alterations in the expression of ABC-transporters. Besides upregulation of MRP1, cMOAT, and MRP3 (Table 3), 30 down-regulation of MDR1 has occurred in these lines (59; this study, Table 3).

Some other cisplatin-resistant lines contain increased levels of *cMOAT* as well, notably 2008/C13\*5.25, 2008/A, A2780/DDP, and HCT8/DDP (Fig. 3, Table 4 and 5).

The combination of cisplatin with doxorubicin resistance in resistant cell lines has been reported before (100, 103) and is also present in two other platin selected lines,

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studied here, PXN94/tetR and KCP-4(-) (Table 5). All other platin-selected lines in Table 5 are doxorubicin sensitive, however. The substrate specificity of the organic anion pumps in the liver canalicular membrane (cMOAT) and in erythrocytes (presumably mainly if not exclusively MRP1) is very similar (91). We therefore expect both pumps to confer similar resistance spectra. We have recently succeeded in obtaining stably transfected kidney cells in which cMOAT is properly routed to the plasma membrane (R. Evers, M.K., and P.B., unpublished). These cells should allow a direct test of the drug resistance spectrum that can be associated with cMOAT overexpression.

Overexpression of CMOAT in cisplatin resistant cell lines was recently also reported by Taniguchi et al. (52). However, in contrast to our results (Table 4) they detected 15 raised cMOAT RNA levels in the KCP-4 and T24/DDP10 cell lines. We do not find this. The level of cMOAT RNA was even decreased in the highly cisplatin resistant KCP-4(+) cells, and in the T24/DDP10 cell line cMOAT RNA is hardly detectable by RNase protection. We also detect no cMOAT 20 protein in these cell lines (Fig. 16). Cross-hybridization of the CMOAT probe used by Taniguchi et al. (52), which contains the coding sequence of the first ATP-binding domain, with RNA transcribed from the other MRP homologs might explain the discrepancy. This underlines the 25 importance of the use of gene specific probes to determine expression of MRP homologs.

In conclusion, our data and those recently published by Ishikawa et al. (35, 36), Fujii et al. (37, 38), Goto et al. (39), Chuman et al. (102), and Taniguchi et al. (52) provide evidence that an organic anion pump, notably cMOAT, contributes to cisplatin resistance by exporting the cisplatin-GSH complex. Elevated GSH levels and synthesis may be required to drive formation of the complex if contact with cisplatin is extended, as is usually the case for cell 35 lines selected for resistance in vitro.

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#### LEGENDS TO THE FIGURES.

Figure la. cDNA sequence of human cMOAT Figure lb. cDNA sequence of rat cMOAT

- Figure 2. (A) Northern blot analysis of 2 μg poly(A)<sup>+</sup> RNA from Wistar rat tissues hybridized to a l-kb *HindIII/AvaII* cDNA fragment of cmoat. RNA was analyzed as described in (the experimental part. Prolonged exposure of the film revealed no detectable expression in other tissues then
- 10 kidney, duodenum, and ileum. (B) Northern blot analysis of 2 μg of poly(A)<sup>+</sup> RNA from Wistar and TR<sup>-</sup> rat liver and hepatocytes hybridized with the same probe as described in (A). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal is shown at the bottom. Molecular size standards are indicated at the right.
- Figure 3. Immunoblot analysis of cmoat and P-glycoprotein in canalicular and basolateral membrane fractions of Wistar and TR<sup>-</sup> rat hepatocytes. Lane 1, Wistar basolateral membranes; Lane 2, Wistar canalicular membranes; Lane 3, TR<sup>-</sup>
- basolateral membranes; Lane 4, TR $^-$  canalicular membranes. Upper panel: the blot was incubated with the monoclonal antibody M $_2$  III-5 directed to cmoat (27). This antibody did not crossreact with the hMRP1 protein as tested in total lysates from the MRP-overexpressing cell line GLC4/ADR (20).
- Lower panel: Immunodetection of P-glycoproteins with the Mab C219 in the same membrane preparations. The 150-kD P-glycoproteins are exclusively expressed in canalicular membranes (22). Differential staining of the two fractions demonstrates the separation of the two membrane domains with
- 30 slight contamination of the basolateral fraction by canalicular membranes. Molecular weight markers are indicated.

Figure 4 Deduced amino acid sequence of the rat cMOAT and alignment with the deduced 70 amino acid sequence of the translated 213-bp putative rat mrp1 cDNA.

Figure 5. Transport of GS-DNP in COS-7 cells transiently transfected with rat cMOAT expression constructs. Closed

circles represent cells transfected with pSVK3-rat cMOAT. Open circles represent cells that have been transfected with a pSVK3 construct with the rat cMOAT cDNA in the reverse orientation and serves as a negative control. The results

Figure 6. Transport of GS-DNP in membrane vesicles prepared from COS-7 cells transiently transfected with rat cMOAT expression constructs in the presence or absence of an ATP regenerating system. Closed squares represent cells

depicted are the mean of three measurements.

- transfected with pSVK3-rat cMOAT. Open squares represent cells that have been transfected with a pSVK3 construct with the rat cMOAT cDNA in the reverse orientation and serves as a negative control. The results depicted are the mean of three measurements.
- Figure 7. Human cMOAT expression in crude lysates from MDCKII derived transfectants. 2 or 20 mg of total protein was size fractionated in a 7.5% polyacrylamide gel containing 0.1% (wt/vol) SDS. After electroblotting, human cMOAT protein was visualized by staining with mAB M2-III-6.
- Protein antibody interaction was detected using the Amersham enhanced chemiluminescence kit (ECL). Lane 1,2 MDCKII cells; lane 3,4 human cMOAT expressing clone MDCKII-216; lane 5,6 human cMOAT expressing clone MDCKII-217. In lanes 1,3 and 5 two micrograms of total protein were loaded and in lanes 2,4 and 6 20 micrograms were loaded.
  - Figure 8. Detection of human cMOAT in MDCKII monolayers by confocal laser-scanning microscopy. A. Indirect immunofluorescence (FITC) picture with mAb M2-III-6 on MDCKII-217 cells. Nucleic acids were detected using
- propidiumiodide (red signal). Top view of the cell layer is shown. B. Optical section perpendicular to the plane of the cell layer.
- Figure 9. Export of GS-DNP from MDCK-II, MDCKII-216, and MDCKII-217 cells. Cells were incubated with [ $^{14}$ C]CDNB (2 mM) in both the apical and basal compartments. Samples were taken at t = 1, 3, 6, 12, and 20 min from both compartments and extracted with ethylacetate. The amount of [ $^{14}$ C]DNP-GS

excreted (in pmol per 2 ml) was measured and plotted. All experiments were done in duplicate and repeated at least twice. Variation between measurements was below 10%. Dotted line: transport to the basal compartment. Continuous line: transport to the apical compartment.

- Figure 10. Deduced amino acid sequence of human cMOAT.

  Predicted transmembrane regions are underlined. Walker A, B, and signature sequence are doubly underlined. Predicted N-glycosylation sites conserved in other cMOAT proteins (rat,
- rabbit) and MRP1 proteins (human, mouse) are indicated with triple asterisks. The triangle indicates the location (amino acid 1066) at which a stop codon is introduced by a C to T transition in DJS cMOAT.
- Figure 11. Immunohistochemical detection of the cMOAT

  protein in human and rat liver using monoclonal antibody
  M2III-6. Sections of a normal human liver (A) and normal rat
  liver (B), which demonstrate the exclusive canalicular
  localization of the protein. In liver sections of the DJS
  patient (C) and the TR<sup>-</sup> rat (D), no canalicular staining is
- observed. Magnifications are 20 x (A, C), and 100 x (B, D). Figure 12. Part of the *cMOAT* cDNA sequence encompassing the mutation which results in the absence of the functional protein in the patient. The normal sequence is depicted on the right. The arrow indicates the site of the mutation at
- codon 1066. This codon normally encodes an arginine residue (CGA), but is changed into a stop-codon (TGA) in the patient. The mutation of C to T eliminates the recognition site for the restriction enzyme TaqI (5'-TCGA-3').
- Figure 13. TaqI digest of a part of the cMOAT cDNA that was obtained with primer combination forwardIV/reverseIV. Lane 1 represents healthy control-, lane 2 the patient cDNA digest. Molecular size markers are indicated on the left in kilo base pairs.

#### Figure 14

Protein alignment of COOH-terminal ends of the five human MRP homologs and human SUR. The alignment was performed with the PILEUP program of GCG (48). The GenBank accession

48

numbers for the proteins used in this comparison are the following: MRP1 - L05628, cMOAT/MRP2 - U49248, MRP3 - U83659, MRP4 -U83660, MRP5 - U83661, SUR - L78207. The nucleotide binding domain specific signature sequence and the Walker A and B motifs are shown in bold. Asterisks above the alignment indicate identical amino acids in at least four of the five MRP proteins.

# Figure 15

RNase protection assays of RNA transcript levels of MDR1, MRP1. cMOAT (MRP2), MRP3, MRP4, and MRP5 in the human non-small-cell lung cancer cell line SW1573/S1 and its doxorubicin selected subline 30.3M. 10 µg total cytoplasmic RNA from each cell line was used per probe. The positions of the protected fragments of MDR1, MRP1-5, and t-actin are

# Figure 16

indicated.

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Immunoblot detection of cMOAT protein in the cell lines analyzed in this paper. Total cell lysates were size fractionated (40 µg per lane) in a 7.5% polyacrylamide gel containing 0.5% SDS. The fractionated proteins were transferred to a nitrocellulose membrane, and cMOAT protein was detected by incubation with monoclonal antibody M2III-5. The size (kDa) and position of molecular weight markers are indicated.

25 Figure 17 A/B.

Nucleotide sequence and amino acid sequence of MRP3.

Figure 18.

Partial MRP4 sequence.

Figure 19 A/B

30 MRP 5 sequences.

#### Table 1

Homology between the COOH-terminal 124 amino acids of the five human MRP homologs and human SUR. Percentages of identity and similarity were determined using the BESTFIT program of GCG (48).

BNSDOCID: <WO\_\_\_\_\_9731111A2\_I\_>

### Table 2

Levels of RNA transcripts of MRP1, cMOAT (MRP2), MRP3, MRP4, and MRP5 in human tissues. RNA expression levels were determined by RNase protection assays with 10 µg total RNA from various human tissues per probe. Expression of t-actin was taken as control for total RNA input. Data for MRP1 RNA levels are from Zaman et al. (52). The relative expression level is indicated by filled circles, very low or undetectable RNA levels by open circles. nd = not determined.

#### Table 3

Characteristics of the doxorubicin-selected cell lines analyzed in this paper. Resistant cell lines were selected by chronically exposing them to the concentrations of

doxorubicin as shown. RNA levels were determined as in Figure 2. The relative expression level is indicated by filled circles, very low expression by \_, and undetectable RNA levels by open circles.

# Table 4

- 20 Characteristics of cell lines selected for resistance to cisplatin, tetraplatin or CdCl<sub>2</sub>. Resistant cell lines were selected by chronically exposing them to the concentrations of drugs as shown. Only A2780/DDP and HCT8/DDP were selected by challenging them 1 h weekly with 50 µM cisplatin. RNA
- levels were determined as in Figure 2. The relative expression level is indicated by filled circles, very low expression by \_, and undetectable RNA levels by open circles. Data for total intracellular glutathione concentrations were obtained from three independently
- isolated cell extracts assayed in three independent experiments using the recycling method of Tietze (56) and presented as the mean GSH  $\pm$  SD.

#### Table 5

IC<sub>50</sub> values and relative resistance factors (RF) of the cell lines analyzed for cisplatin and doxorubicin. IC<sub>50</sub> data were obtained from clonogenic survival assays with continuous exposure to drugs. The relative resistance factor was

50

determined by dividing the  $IC_{50}$  of each resistant cell line by the  $IC_{50}$  of the corresponding parental cell line. Also shown are the levels of RNA transcripts of MRP1 and cMOAT, taken from Table 3 and 4.

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  20 follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G,
  Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P,
  Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y,
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Table 1

Homology between COOH-terminal 124 amino acids of human MRP homologs and human SUR (% similarity / % identity)

	MRP1	cMOAT	MRP3	MRP4	MRP5	HSUR
MRP1	100 / 100					
cMOAT	19 / 68	100 / 100				
MRP3	86 / 75	79 / 62	100 / 100			
MRP4	75 / 60	75 / 54	71/53	100 / 100		
MRP5	75 / 55	75 / 53	71 / 51	73 / 57	100 / 100	
HSUR	69 / 48	68 / 46	66 / 46	69 / 48	67 / 45	100 / 100

Table 2

	MRP1	cMOAT	MRP3	MRP4	MRP5
Qui'l		0	•	•	:
Kidoev	:	•	•	•	:
Riadder	:	0	•	•	:
Spleen	:	С	•	0	:
Mamma	<b>1</b> 0	э	С	5	2
Salivary gland	Ę	0	၁	O	:
Thyroid	:	С	С	С	•
Testis	:	0	c	С	:
Nerve	•	•	2	c	:
Stomach	:	o	•	С	:
Liver	0	•	•	0	•
Gall bladder	:	ъ	5	•	•
Duodentim	:	:	:	힏	P
Colon	:	o	:	3	•
Adrenal gland	:	o	:	c	•
Skeletal muscle	:	э	0	0	•
Heart	•	Ç	c	c	:
Brain	•	o	С	С	•
Placenta	:	O	0	0	•
Ovarium	:	٥	0	0	•
Pancreas	•	Э	•	3	•
Tone	90	0	•	•	:

nd = not determined

0 = no expression

• . • • • • = tow to high expression

Characteristics of the doxorubicin-selected MDR cell lines analyzed

1 1 1					/111	CIDADI CANU		
Cell line	Source	orug used ror selection	MDR1	MRP1	cMOAT	MRP3	MRP4	MRP5
	Non small cell lung cancer		€	•	€	€	•	•
30.3M		dox ( 30 nM)	⊕	:	:	:	•	•
1R50b		dox ( 50 nM)	o	•	:	•	•	•
2R50		dox ( 50 nM)	С	:	⊕	⊕	•	•
3R80		dox ( 80 nM)	o	•	⊕	:	•	•
2R120		dox ( 120 nM)	•	:	:	:	•	•
2R160		dox ( 160 nM)	:	:	:	•	•	•
18500		dox ( 500 nM)	•	•	:	€	•	•
COR-L23	Non small cell lung cancer		c	•	၁	0	•	<b>⊕</b>
COR-L23/R		dox ( 368 nM)	5	•	0	0	•	⊕
910	Small cell lung cancer		c	•	o	0	•	•
GLC,/ADR		dox (1160 nM)	c	:	С	С	•	•
MOR/P	Adeno lung carcinoma		э	•	•	:	•	:
MOR/R		dox ( 368 nM)	0	•	:	:	•	:
нгео	Leukemia carcinoma		o	•	0	0	•	<b>⊕</b>
HL60/ADR		dox ( 186 nM)	၁	:	0	0	•	<b>⊕</b>

= no expression

= very low expression

•••• = low to high expression

Table 3

Characteristics of the cell lines selected for resistence to cisplatin, tetraplatin or CdCl,

	90	Drug used for							
00095		selection	MDR1	MRP1	cMOAT	MRP3	MRP4	MRP5	(nmol/mg protein)
DDP5 DDP7		The state of the s	c	•	<b>•</b>	•	•	•	11.4 ± 2.4
24/DDP5 24/DDP7	Bladder carcinoma		. •	•	• €	0	•	•	26.8 ± 0.1
24/DDP7					• €	⊕	•	•	65.5 ± 1.8
124/DDP10		CDDP ( 6.7 µM)		: :	• ⊕	⊕	•	:	59.5 ± 3.3
			•	:	€	⊕	⊕	:	33.5 ± 1.5
2008 Ovar	Ovarium carcinoma	44.00	, c	:	•	•	<b>⊕</b>	:	$113.2 \pm 16.6$
2008/C13*5.25		COURT 19:0 pini)	. •	:	€	•	⊕	:	48.5 ± 5.4
2008/MT 2008/A		CDDP ( 0.5 µM)	, c	:	•	⊕	⊕	:	124.7 ± 18.6
			c	•	<del>-</del>	÷	⊕	:	133 1 0.9
A2780 Ovar A2780/DDP	Ovarium carcinoma	CDDP (50.0 µM)	٤	•		c	⊕	:	26.5 ± 2.4
			€	:	э	S	•	:	40.2 ± 5.6
PXN94 Ovai PXN94/tetA	Ovarium carcinoma	Tetraplatin (2.0 $\mu$ M)	· <del>-</del>	:	c	Ξ	•	:	91.8 ± 8.8
				•	•	•	•	•	17.7 ± 2.2
HCT8.DDP	Colon carcinolna	CDDP (50.0 µM)	•	:	:	:	•	:	70.2 ± 6.8
			5	•	<b>e</b>	s	9	:	5.2 ± 0.2
GC127 GC127cisR	Lesiis Carcinoma	CDDP ( 4.0 µM)	c	•	⊕	ε	⊕	:	9.2 ± 3.5
1 0 0 7	Fuidermoid carcinoma		c	:	:	:	⊕	•	36.7 ± 0.9
_		CDDP (23.3 uM)	•	:	:	:	⊕	:	72.7 ± 8.9
KCP-4(+)		CDDP ( 6.7 µM)	P.	50	⊕	ē	뒫	٦	158.4 ± 13.3

= no expression

= very low expression = low to high expression - & · P:

= not determined

Table 4

Table 5

	Cisplat	in	Doxorul	picin	RNA	levels
Cell line	IC <sub>so</sub> (nM)	RF	IC <sub>so</sub> (nM)	RF	MRP1	cMOAT
S1	144		13.8	<del></del>	•	÷
30.3M	500	3.5	51.1	3.7	••	• • •
1R50b	194	1.5	84.2	6.1	•	• •
2R50	115	0.9	69.0	5.0	••	<del>-5)</del>
3R80	nd	nd	75.9	5.5	•	<del>-</del>
2R120	313	2.2	345	25	••	• •
2R160	600	4.2	1380	100	••	•••
1R500	260	1.7	3450	250	•	••
T24	825		6.5		•	Э
T24/DDP5	2200	2.7	5.5	0.8	•	€
T24/DDP7	1800 .	2.2	3.0	0.5	••	Ð
T24/DDP10	8000	9.7	6.5	1.0	••	∌
2008	340	•	57.6	-	••	⊕
2008/C13*5.25	3000	8.8	43.2	0.8	• •	•
2008/MT	210	0.6	54.4	0.9	• •	⊕
2008/A	680	2.0	36.8	0.6	••	•
A2780	430		5.2		•	⊕
A2780/DDP	4300	10.0	6.2	1.2	•	••••
PXN94	580	•	4.2	-	••	၁
PXN94/tetR	2400	4.1	11.0	2.6	••	o
нств	1900		90.0	•	•	•
HCT8/DDP	4800	2.5	95.0	1.1	••	•••
GCT27	288		3.0	-	•	<b>⊕</b>
GCT27cisR	2100	7.3	3.1	1.0	•	⊕
(B-3-1	380		11.0	-	••	•••
(CP-4(-)	700	1.8	43.0	3.9	••	•••
(CP-4(+)	22400	58.9	8.0	0.7	nd	<b>⊕</b>

66

#### CLAIMS

- 1. A nucleic acid comprising a sequence encoding at least a part of a member of a family of organic anion transporters, with the exclusion of mammalian Multidrug Resistance Associated Protein, said nucleic acid comprising at least a gene family specific fragment of one of the sequences of fig.la or fig.lb or the complement thereof, or a sequence having at least 55%, preferably 70%, in particular 90% homology therewith.
- 2. A nucleic acid according to claim 1 encoding at least a part of a mammalian member of said family.
  - 3. A nucleic acid according to claim 2 encoding at least a part of a human member of said family.
  - 4. A nucleic acid and/or its complement having at least part of the sequence of fig.lA and encoding a protein having human Canalicular Multispecific Organic Anion Transport protein or similar activity or antigenicity.
  - 5. A nucleic acid and/or its complement having at least part of the sequence of fig.1b and encoding a protein having rat Canalicular Multispecific Organic Anion Transport
- 20 protein activity or antigenicity.
  - 6. A nucleic acid encoding rat Canalicular Multispecific Organic Anion Transport protein or human Canalicular Multispecific Organic Anion Transport protein.
- A vector comprising a nucleic acid according to anyone
   of the foregoing claims and suitable means for replication,
   transduction and/or expression of said nucleic acid.
  - 8. A vector according to claim 7 further comprising a gene encoding a therapeutically beneficial protein.
- 9. A vector according to claim 7 or 8 further comprising a gene encoding glutathion elevating activity.
  - 10. A vector according to claim 9 wherein the gene encodes at least a functional part of a gamma glutamyl cysteine synthetase or a UDP-glucuronosyltransferase.

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- 11. A vector according to claim 7 wherein the therapeutically beneficial protein is another multidrugresistance related protein such as MDR1.
- 12. A cell comprising a nucleic acid or a vector according to anyone of the foregoing claims.
- 13. A cell according to claim 12 further comprising a vector encoding glutathion elevating activity.
- 14. A cell according to claim 12 or 13 provided with other resistance related proteins such as MDR1.
- 10 15 A method for providing cells with Canalicular Multispecific Organic Anion Transport protein activity, comprising transducing said cell with a nucleic acid or a vector according to anyone of claims 1-11.
  - 16. A method for enhancing Canalicular Multispecific
- Organic Anion Transport protein activity of cells according to claim 15, comprising increasing the intracellular level of glutathion, glucuronide and/or sulphate.
  - 17. A method for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according
- to claim 15, comprising enhancing the conjugating capacity and/or the compelxing activity of said cell for sulphate, glutathion, glucuronide and the like.
  - 18. A method for reducing Canalicular Multispecific Organic Anion Transport protein activity and/or the multidrug
- resistance of a cell comprising providing said cell with an antisense construct of a nucleic acid or a vector according to anyone of claims 1-11.
  - 19. A method according to claim 18, further comprising providing the cell with an antisense construct derived from another multidrug resistance related protein such as MDR1.
  - 20. A protein encoded by a nucleic acid according to anyone of claims 1-6 or obtainable by expression of a vector according to anyone of claims 7-11.
  - 21. A protein having Canalicular Multispecific Organic
- 35 Anion Transport protein activity or Canalicular Multispecific Organic Anion Transport protein specific

68

antigenicity comprising at least part of the sequence of fig.4 or another mammalian equivalent thereof.

- 22. Use of a nucleic acid according to anyone of claims 1-6 or a protein according to claim 20 or 21 in the diagnosis of Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport
  - protein.

    23. Use of a nucleic acid according to anyone of claims 1-6 or a protein according to claim 20 or 21 in the treatment of Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport
  - protein.

    24. Use of a nucleic acid according to anyone of claims 1-6 as a selectable marker gene.

BNSDOCID: <WO\_\_\_\_\_9731111A2\_I\_>

1	AGGATAATTC	CIGITICCACI	TTCTTTGAT	AAACAAGTA	A AGAAGAAACI
51	ACACAATCAT	ATTAATAGAA	A GAGTetTCGt	TCCAGACgC	A GECCAGGAA
101	CATGCTGGAG	AAgTTCTGCA	ACTCTACTT	' tTGGAATTC	C TCATTCcTgg
151	ACAGTCCGGA	GGCAGACCTG	CCacTTTGTt	TtGAGCAAA	TGTTcTGGT
201	TGGATTCCcT	TGGGCTTCcT	ATGGCTCCTG	GCCCcctGgC	agcTTcTCcA
251	cGtGtAtAAA	tCCaggACCA	AGAGATCcTc	TACCACCAA	CTCtaTcTTG
301	cTAaGCaGGT	ATTcGttGgT	' tTtCttcTTA	TtcTAgCAgc	: CATAGAGCEG
351	GCCCTTGTAC	TCACAGAAGA	CTCTGGACAA	GCCACAGTCC	: cTGCTGTtCG
401	ATATACCAAT	CCAAGCCTCT	ACCTAGGCAC	AtGGeTCCTG	GTTTTGCTGA
451	TCCAATACaG	cAGACAATGG	TGTGTACAGA	AAAACTCCTG	GTtCCTGtCC
501	ctattctgga	TTCTCTcGAT	ACTCtGtGGC	ACTTTCCAaT	TTCAGACTCt
551	gATccGGAca	CTCTTACAGG	GTGACAAtTc	TAATCTAGCC	TACTCcTGcC
601	TGTTCTTCAt	CtCcTAcGGa	tTcCaGATCc	TGATCCTGAT	CLTTTCAGCA
651	TTTTCAGAAA	ATAATGAGTC	ATCAAATAAT	CCATCATCCA	TAGCTTCALT
701	CCTGAgTAgC	ATTACCTACA	GCTGGTATGA	CAgCaTCATT	CTGAAAgGcT
751	ACaAgCgTcC	TCTGACACTC	gAgGATgTcT	GGGAAgttGA	TGAAgAgATg
801	AAAACCAAGA	CATTAGTGAG	CAAGTTTGaa	ACGCACATGA	AGAGAGAGCT
851	GCAGAAAGCC	AGGCGGGCAC	TCCAGAGACG	GCAGGAGAAG	AGCTCCCAGC
901	AGAACTCTGG	AgCCAGGCTG	CCTGGCTTGA	ACAAGAATCA	GAGTCAAAGC
951	CAAGATGCCC	TTGTCCTGGA	AGATGTTGAA	AAGAAAAAA	AGAAGTCTGG
1001	GACCAAAAA	GATGTTCCAA	AATCCTGGTT	GATGAAgGCt	CTGTTCAAAA
1051	CTTTCTACAT	GGTGCTCCTG	AAATCaTTCC	TACTGAAgCT	AGTGAATGAC
1101	aTCTTCACgT	TTGTGAGTCC	TCAgCTGCTG	AAALTGCTGA	TCTCCtTTGC
1151	AAGTGAcCGt	GACACATATT	TGTGGATTGG	ATATCTCTGT	GCaATCCTCT
1201	TATTCACTGC	GGCTCTCATT	CAGTCTTTCT	GCCTTCAgTG	TTaTTTCCAA
1251	CTGTGCTTCA	AGCTGGGTGT	AAAAgTACGg	ACAGCLATCa	tgGcTTcTGT
1301	ATATAAGAAG	GCATTGACCC	TATCCAACTT	GGCCaGGAAG	GAGTACACCG
1351	tTGGAGAAAC	AGTGAACCTG	ATGTCTGTGG	ATGCCCAGAA	GcTCATGGAT
1401	GTGACCAACT	TCATGCACAT	GCTGTGGTCA	AGTGTTCTAC	AGATTGTCTT FIG 1a

2/36

1451	ATCTATCTTC	TTCCTAtGGA	GAGAGTTGGG	ACCCTCAGTC	TTAGCAGGTG
1501	TTGGGGTGAT	GGTGCTTGTA	ATCCCAATTA	ATGCGATACT	GTCCACCAAG
1551	AGTAAGACCA	TTCAGGTCAA	AAATATGAAG	AATAAAGACA	AACGTTTAAA
1601	GATCATGAAT	GAGATTCTTA	GTGGAATCAA	GATCCTGAAA	TATTTTGCCT
1651	GGGAACCTTC	ATTCAGAGAC	CAAGTACAAA	ACCTCCGGAA	GAAAGAGCTC
1701	AAGAACCTGC	TGGCCTTTAG	TCAACTACAG	TGTGTAGTAA	TATTCGTCTT
1751	CCAGTTAACT	CCAGTCCTGG	TATCTGTGGT	CACATTTTCT	GTTTATGTCC
1801	TGGTGGATAg	CAACAATATT	TEGGATGCAC	AAAAGGCCTT	CACCTCCATT
1851	ACCCTCTTCA	ATATCCTGCG	CTTCCCCCTG	AGCATGCTTC	CCATGATGAT
1901	CTCCTCCATG	CTCCAGGCCA	GTGTTTCCAC	AGAGCGGCTA	GAGAAGTACT
1951	TGGGAGGGGA	TGACTTGGAC	ACATCTGCCA	TTCGACATGA	CTGCAATTTT
2001	GACAAAGCCA	TGCAGTTTTC	TGAGGCCTCC	TTTACCTGGG	AACATGATTC
2051	GGAAGCCACA	GTCCGAGATG	TGAACCTGGA	CATTATGGCA	GGCCAACTTG
2101	TGGCTGTGAT	AGGCCCTGTC	GGCTCTGGGA	AATCCTCCTT	GATATCAGCC
2151	ATGCTGGGAG	AAATGGAAAA	TGTCCACGGG	CACATCACCA	TCAAGGGCAC
2201	CACTGCCTAT	GTCCCACAGC	AGTCCTGGAT	TCAGAATGGC	ACCATAAAGG
2251	ACAACATCCT	TTTTGGAACA	GAGTTTAATG	AAAAGAGGTA	CCAGCAAGTA
2301	CTGGAGGCCT	GTGCTCTCCT	CCCAGACTTG	GAAATGCTGC	CTGGAGGAGA
2351	TTTGGCTGAG	ATTGGAGAGA	AGGGTATAAA	TCTTAGTGGG	GGTCAGAAGC
2401	AGCGGATCAG	CCTGGCCAGA	GCTACCTACC	AAAATTTAGA	CATCTATCTT
2451	CTAGATGACC	CCCTGTCTGC	AGTGGATGCT	CATGTAGGAA	AACATATTTT
2501	TAATAAGGTC	TTGGGCCCCA	ATGGCCTGTT	GAAAGGCAAG	ACTCGACTCT
2551	TGGTTACACA	TAGCATGCAC	TTTCTTCCTC	AAGTGGATGA	GATTGTAGTT
2601	CTGGGGAATG	GAACAATTGT	AGAGAAAGGA	TCCTACAGTG	CTCTCCTGGC
2651	CAAAAAAGGA	GAGTTTGCTA	AGAATCTGAA	GACATTTCTA	AGACATACAG
2701	GCCCTGAAGA	GGAAGCCACA	GTCCATGATG	GCAGTGAAGA	AGAAGACGAT
2751	GACTATGGGC	TGATATCCAG	TGTGGAAGAG	ATCCCCGAAG	ATGCAGCCTC
2801	CATAACCATG	AGAAGAGAGA	ACAGCTTTCG	TCGAACACTT	AGCCGCAGTT
2851	CTAGGTCCAA	TGGCAGGCAT	CTGAAGTCCC	TGAGAAACTC	CTTGAAAACT
2901	CGGAATGTGA	ATAGCCTGAA	GGAAGACGAA	GAACTAGTGA	AAGGACAAAA
2951	ACTAATTAAG	AAGGAATTCA	TAGAAACTGG	AAAGGTGAAG	TTCTCCATCT
3001	ACCTGGAGTA	CCTACAAGCA	ATAGGATTGT	TTTCGATATT	CTTCATCATC

	3/36
3051	CTTGCGTTTG TGATGAATTC TGTGGCTTTT ATTGGATCCA ACCTCTGGCT
3101	CAGTGCTTGG ACCAGTGACT CTAAAATCTT CAATAGCACC GACTATCCAG
3151	CATCTCAGAG GGACATGAGA GTTGGAGTCT ACGGAGCTCT GGGATTAGCC
3201	CAAGGTATAT TTGTGTTCAT AGCACATTTC TGGAGTGCCT TTGGTTTCGT
3251	CCATGCATCA AATATCTTGC ACAAGCAACT GCTGAACAAT ATCCTTCGAG
3301	CACCTATGAG ATTTTTTGAC ACAACACCCA CAGGCCGGAT TGTGAACAGG
3351	TTTGCCGGCG ATATTTCCAC AGTGGATGAC ACCCTGCCTC AGTCCTTGCG
3401	CAGCTGGATT ACATGCTTCC TGGGGATAAT CAGCACCCTT GTCATGATCT
3451	GCATGGCCAC tCCTGTCTTC ACCATCATCG TCATTCCTCT tGGCAttAtt
3501	tatgeatotg tecagatgtt teatgegtct acctcccgcc agetgaggcg
3551	TCTGGACTCT GTCACCAGGT CCCCAATCTA CTCTCACTTC AGCGAGACCG
3601	TATCAGGTTT GCCAGTTATC CGTGCCTLTG AGCACCAGCA GCGATTTCTG
3651	AAACACAATG AGGAGAGGAL TGACACCAAC CAGAAAtGTG TCTTTTCCTG
3701	GATCACCTCC AACAGGEGGC TTGCAATTCG CCTGGAGCTG GTTGGGAACC
3751	TGACTGTCTT CTTTTCAGCC LTGATGATGG TTATTTALAG AGATACCCTA
3801	AGEGGGGACA CTGTTGGCTT TGTTCTGTCC AATGCACTCA ATATCACACA
3851	AACCCTGAAC TGGCTGGEGA GGATGACATC AGAAATAGAG ACCAACATTG
3901	tGGcTGTtGA GCGAATAACt GAGtACACAA AAGTGGAAAA TGAGGCACCC
3951	TGGGTGAGEG ATAAGAGGCC ECCGCCAGAT TGGGCCCAGCA AAGGCAAGAT
4001	CCAGETTAAC AACTACCAAG TGCGGTACCG ACCTGAGCTG GATCTGGTCC
4051	tCaGAgGGAT CACTTGTGAC ATCGGTAGCA TGGAGAAGAt TGGTGTGGTG
4101	GGCagGAcAG gAgctGGAAa gTcatcCCTC ACAAactGcC TcTTcaGaAT
4151	CTTAGAGGCT GCCGGTGGTC AGATTATCAT TGATGGAGTA GATATTGCTT
4201	CCATTGGGCT CCACGACCTC CGAGAGAGC TGACCATCAT CCCCCAGGAC
4251	CCCATeCTGT TeteTGGAAG cCTGAGGATG AATCTcGACC cTTTCAACAA
4301	CTACTCAGAT GAGGAGATTT GGAAGGCCTT GGAGCTGGCT CACCTCAAGT
351	CTTTTGTGGC CAGCCTGCAA CTTGGGTTAT CCCACGAAGT taCAGAGGCT
401	gGTgGCAACC TGAGCATAgG CCAgAggeag CTGCTGTGeC TGGGCAGGGe
451	TCTGCTTCGG AAATCCAAGA TCCTGGTCCT GGATGAGGCC ACTGCTGCGG
501	TGGATCTAGA GACAGACAAC CTCATTCAGA CGACCATCCA AAACGAGTTC
551	GCCCACTGCA CAGTGATCAC CATCGCCCAC AGGCTGCATA CCATCATGGA
601	CAGTGaCaAg GTaATGGtCC tAGaCaAcgG GaAgaTtAtA gAgTACGGCa

651	gCCcTGaAGA	acTgCtaCAA	ATCCCTGGAC	CCTTTEACET	TATGGCTAAG
701	GaAgCTgGCA	TTGAGAATGT	gAaCAgCaCa	aAAtTcTagc	agaaggcCCC
751	ATgGGtTaga	AAagGactat	AagaatAATT	tCtTAtttAa	ttttAtttt
801	tataaAAtaC	aGaataCata	CaaaagtgTg	taTaaAATGt	ACgTTTTaaa
851	aAAGGaTAag	TgaacACCCa	TGAACctact	ACCcAgGTTA	AgaaaataAa
901	tgTCaCCAGg	TactTGAgAA	ACCcctcgAt	TGTCtACcTC	gATCgTactT
951	CCtTGcTACC	cacccctccc	AGGgacAAcC	AcTgTCctGA	attTcaCgAt
5001	AATtattCCt	tTGCCTtTca	tTTCTGTTTT	ATCACCTTTG	TATGTATCTT
5051	TAAACAACAT	ATACCCTTTT	TTACTTATGT	AAATGGACTG	ACTCATACTG
5101	CATACATCTT	CTATGACTTG	AtTCTTTTGT	tCAAtAttat	AtctGagatT
5151	CATCCATGGT	GATGCAAATA	GGTGCATTAt	TTTTTTCAC	TGCTCTGTAg
5201	TCTGGCattg	tatGaATacA	gcacaatgtA	tcagTtttaa	Tattggggat
5251	catTagcatt	atTctcaggt	tttTaaaaAt	tAtaagcAgt	actactatgg
301	AaaGAAAnTC	TGGTCCTACA	TCTCCCTGGC	ACATATGTAA	GAGTTTCnCn
5351	AGGGTALAAC	Ctaggaatgg	AGGGTATGAA	CATGTTTACA	TGCACAAACT
5401	AGCEGATGCC	AAACTGGTAA	TGCCAACTGA	AAACAETGCT	GTCAAtCTGA
5451	TGAATATGAA	TTGATGTATC	AATGAGAATT	TCATTTGCAT	TTCCCTAGTA
5501	TCTATTGGGG	ATGAATATAT	TTTCATGLTT	CTGGGGCATT	TGCATTTCCA
5551	Cutut Catutalanala	AAATATTGTG	TCTGATAWTT	vTnnAT	

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1	TGCACTTTAA	CATCTGCTTT	CCCAGAGGAA	AAAGTAAAGG	AGAAACAGTA
51	CAATCATAGA	AGAGTCTTCG	TAACAGAAGC	GCGAGGAGAG	CATTATGGAC
101	AAGTTCTGCA	ACTCTACTTT	TTGGGATCTC	TCATTACTGG	AAAGTCCAGA
151	GGCTGACCCG	CCTCTTTGTT	TTGAGCAAAC	TGTTCTGGTG	TGGATTCCCT
201	TGGGCTTTCT	TTGGCTCCTG	GCTCCTTGGC	AACTTTACAG	CGTGTACAGA
251	TCCAGGACCA	AGAGATCTTC	ТАТААССААА	TTCTACCTTG	CCAAGCAGGT
301	GTTCGTCGTG	TTTCTTCTTA	TTTTAGCAGC	CATAGACCTG	TCTCTTGCGC
351	TCACAGAAGA	TACTGGACAA	GCCACAGTTC	CTCCTGTCAG	ATATACGAAT
401	CCAATCCTCT	ACCTGTGCAC	ATGGCTCCTG	GTTTTGGCAG	TCCAGCACAG
451	CAGGCAATGG	TGTGTACGAA	AGAACTCTTG	GTTCCTGTCT	CTGTTCTGGA
501	TCCTCTCGGT	CTTATGCGGC	GTATTCCAGT	TTCAGACTCT	GATACGAGCA
551	CTCCTGAAGG	ACAGCAAGTC	CAACATGGCC	TACTCCTACC	TGTTCTTCGT
601	CTCCTACGGT	TTCCAGATTG	TCCTCCTGAT	TCTTACAGCC	TTTTCAGGAC
651	CAAGTGACTC	AACACAAACT	CCATCAGTCA	CGGCTTCCTT	TCTGAGTAGC
701	ATTACATTTA	GTTGGTATGA	CAGGACTGTT	CTGAAAGGTT	ACAAGCATCC
751	ACTGACACTA	GAAGATGTCT	GGGATATCGA	TGAAGGGTTT	AAAACAAGGT
801	CAGTCACCAG	CAAGTTTGAG	GCGGCCATGA	CAAAGGACCT	GCAGAAAGCC
851	AGGCAGGCTT	TTCAGAGGCG	GCTGCAGAAG	TCCCAGCGGA	AACCTGAGGC
901	CACACTACAC	GGACTGAACA	AGAAGCAGAG	TCAGAGCCAA	GACGTTCTCG
951	TCCTGGAAGA	AGCGAAAAAG	AAGTCTGAGA	AGACCACCAA	AGACTATCCC
1001	AAATCGTGGT	TGATCAAGTC	TCTCTTCAAA	ACCTTCCACG	TAGTGATCCT
1051	GAAATCATTT	ATACTGAAAT	TAATACATGA	CCTTTTGGTG	TTTCTGAATC
1101	CTCAGCTGCT	GAAGTTGCTG	ATCGGTTTCG	TGAAGAGCTC	TAACTCATAC
1151	GTGTGGTTTG	GCTATATCTG	TGCAATCCTA	ATGTTTGCTG	TGACTCTCAT
1201	CCAATCTTTC	TGCCTTCAGT	CTTACTTTCA	ACATTGTTTT	GTGTTGGGAA
1251	TGTGCGTACG	GACAACCGTC	ATGTCTTCGA	TATATAAGAA	GGCATTGACC
1301	CTATCTAACT	TGGCTAGGAA	GCAGTACACC	ATTGGAGAGA	CGGTGAACTT
1351	GATGTCTGTA	GATTCCCAGA	AGCTAATGGA	TGCGACCAAC	TACATGCAGT
1401	TGGTGTGGTC	AAGTGTTATA	CAGATTACTT	TGTCCATCTT	CTTCCTGTGG FIG. 1b

1451	AGAGAGTTGG	GACCGTCCAT	CTTAGCAGGT	GTTGGGGTTA	TGGTTCTCCT
1501	AATCCCAGTT	AATGGAGTTC	TGGCTACCAA	GATCAGAAAT	ATTCAGGTCC
1551	AAAATATGAA	GAATAAAGAC	AAACGTTTAA	AAATCATGAA	TGAGATTCTC
1601	AGTGGAATCA	AGATCCTGAA	ATACTTTGCC	TGGGAACCTT	CATTTCAAGA
1651	GCAAGTCCAG	GGCATTCGGA	AGAAAGAACT	CAAGAACTTG	CTGCGGTTCG
1701	GCCAGCTGCA	GAGTCTGCTG	ATCTTCATTT	TACAGATAAC	TCCAATCCTG
1751	GTGTCTGTGG	TCACATTTTC	TGTCTATGTC	CTGGTGGATA	GCGCCAATGT
1801	TTTGAATGCG	GAGAAGGCAT	TTACCTCCAT	CACCCTCTTC	AATATCCTAC
1851	GCTTCCCTCT	GTCCATGCTT	CCCATGGTGA	CCTCATCGAT	CCTCCAGGCC
1901	AGTGTTTCTG	TGGACCGGCT	GGAGAGGTAT	TTGGGAGGAG	ACGATTTAGA
1951	CACATCTGCC	ATTCGCGCCG	TCAGCAATTT	TGATAAAGCT	GTGAAGTTTT
2001	CAGAGGCCTC	TTTTACTTGG	GACCCGGACT	TGGAAGCCAC	AATCCAAGAT
2051	GTGAACCTGG	ACATAAAGCC	AGGCCAACTG	GTGGCTGTGG	TGGGCACTGT
2101	AGGCTCTGGG	AAATCCTCTT	TGGTATCAGC	CATGCTGGGA	GAAATGGAAA
2151	ACGTTCACGG	GCACATCACC	ATCCAGGGAT	CCACAGCCTA	TGTCCCTCAG
2201	CAGTCCTGGA	TTCAGAATGG	AACCATCAAA	GACAACATCC	TGTTTGGGTC
2251	CGAATACAAT	GAAAAGAAGT	ACCAGCAAGT	TCTCAAAGCA	TGCGCTCTCC
2301	TCCCAGACTT	GGAAATATTG	CCTGGAGGAG	ACATGGCTGA	GATCGGAGAG
2351	AAGGGGATAA	ATCTCAGTGG	TGGTCAGAAG	CAGCGAGTCA	GCCTGGCCAG
2401	AGCTGCCTAT	CAAGATGCTG	ACATCTATAT	TCTGGACGAT	CCCCTGTCGG
2451	CTGTGGATGC	TCATGTGGGA	AAACACATTT	TCAACAAGGT	TGTGGGCCCC
2501	AACGGCCTGT	TGGCTGGCAA	GACGAGAATC	TTTGTTACTC	ATGGTATTCA
2551	CTTCCTTCCC	CAAGTGGATG	AGATTGTAGT	TCTGGGGAAA	GGCACCATCT
2601	TAGAGAAAGG	ATCCTATCGT	GACCTGTTGG	ACAAGAAGGG	AGTGTTTGCT
2651	AGGAACTGGA	AGACCTTCAT	GAAGCATTCA	GGGCCTGAAG	GAGAGGCCAC
2701	AGTCAATAAT	GACAGTGAGG	CGGAAGACGA	CGATGATGGG	CTGATTCCCA
2751	CCATGGAGGA	AATCCCTGAG	GATGCAGCTT	CCTTGGCCAT	GAGAAGAGAA
2801	AATAGTCTTC	GCCGTACACT	GAGCCGCAGC	TCTAGGTCCA	GCAGCCGACG
2851	TGGGAAGTCC	CTCAAAAACT	CCTTGAAGAT	TAAAAATGTG	AATGTCTTGA
2901	AGGAGAAGGA	AAAAGAAGTG	GAAGGACAAA	AACTAATTAA	GAAAGAATTT
2951	GTGGAAACCG	GGAAGGTCAA	GTTCTCCATC	TACCTGAAGT	ATCTACAGGC
3001	AGTAGGGTGG	TGGTCCATAC	TTTTCATCAT	CCTTTTCTAC	GGATTGAATA

3051	ATGTTGCTTT	TATCGGCTCT	AACCTCTGGC	TGAGTGCTTG	GACCAGTGAC
3101	TCTGACAACT	TGAATGGGAC	CAACAATTCG	TCTTCTCATA	GGGACATGAG
3151	AATTGGGGT	TTTGGAGCTC	TGGGATTAGC	: ACAAGGTATA	TGTTTGCTTA
3201	TTTCAACTCT	GTGGAGCATA	TATGCTTGCA	GAAATGCATC	AAAAGCTTTG
3251	CACGGGCAGC	TGTTAACCAA	CATCCTCCGG	GCACCCATGA	GGTTTTTTGA
3301	CACAACTCCC	ACAGGCCGGA	TTGTGAACAG	ATTTTCTGGT	GATATTTCTA
3351	CTGTGGACGA	CTTGCTCCCC	CAGACACTTC	GAAGCTGGAT	GATGTGTTTC
3401	TTTGGCATCG	CTGGCACTCT	TGTCATGATC	TGCATGGCCA	CCCCAGTCTT
3451	CGCTATCATC	ATCATTCCTC	TCAGCATTCT	TTATATTTCG	GTGCAGGTTT
3501	TTTATGTGGC	TACTTCCCGC	CAGCTGAGAC	GGTTGGATTC	TGTCACCAAA
3551	TCTCCGATCT	ATTCTCACTT	CAGTGAGACT	GTCACAGGTT	TGCCCATTAT
3601	CCGTGCCTTT	GAGCACCAGC	AGCGATTTCT	AGCTTGGAAT	GAGAAGCAGA
3651	TTGACATCAA	CCAGAAATGT	GTCTTTTCCT	GGATTACCTC	CAACAGGTGG
3701	CTTGCAATTC	GGCTGGAGCT	GGTTGGAAAC	TTGGTCGTCT	TCTGTTCCGC
3751	CTTGCTGCTG	GTTATTTATA	GAAAAACCTT	AACCGGGGAC	GTTGTGGGCT
3801	TTGTTCTGTC	CAACGCCCTC	AATATCACAC	AAACCTTGAA	CTGGCTAGTG
3851	AGGATGACGT	CAGAAGCAGA	GACCAACATT	GTGGCAGTTG	AGCGAATAAG
3901	TGAATACATA	AATGTAGAGA	ATGAGGCGCC	CTGGGTGACT	GACAAGAGGC
3951	CTCCGGCAGA	CTGGCCCAGA	CATGGTGAGA	TCCAGTTTAA	CAACTATCAA
4001	GTGCGGTATC	GGCCGGAGCT	GGATCTGGTA	CTGAAAGGGA	TCACTTGTAA
4051	CATCAAGAGC	GGAGAGAAGG	TCGGCGTAGT	GGGCAGGACT	GGGGCTGGGA
4101	AATCATCCCT	CACAAACTGC	CTCTTCAGAA	TCTTAGAGTC	TGCGGGGGGC
4151	CAGATCATCA	TTGATGGGAT	AGATGTTGCC	TCCATTGGAC	TGCACGACCT
4201	TCGAGAGAGG	CTGACCATCA	TTCCCCAGGA	CCCCATTTTG	TTCTCGGGGA
4251	GTCTGAGGAT	GAATCTCGAC	CCTTTCAACA	AATATTCAGA	TGAGGAGGTT
4301	TGGAGGGCCC	TGGAGTTGGC	TCACCTCAGA	TCCTTTGTGT	CTGGCCTACA
4351	GCTTGGGTTG	TTATCCGAAG	TGACAGAGGG	TGGTGACAAC	CTGAGCATAG
4401	GGCAGAGGCA	GCTCCTATGC	CTGGGCAGGG	CTGTGCTTCG	AAAATCCAAA
4451	ATCCTGGTCC	TGGATGAAGC	CACGGCTGCA	GTGGATCTGG	AGACGGATAG
4501	CCTCATTCAG	ACGACCATCC	GAAAGGAGTT	CTCCCAGTGC	ACGGTCATCA
4551	CCATCGCTCA	CAGGCTGCAC	ACCATCATGG	ACAGTGACAA	GATAATGGTC
4601	CTAGACAACG	GGAAGATTGT	CGAGTATGGC	AGTCCTGAAG	AACTGCTGTC

651	CAACAGAGGT	TCCTTCTATC	TGATGGCCAA	GGAAGCCGGC	ATTGAAAATG
701	TGAATCACAC	AGAGCTCTAG	CAGCTGGTTC	CGTGGCTGGC	GGACTATAAG
1751	AACAGTTTCT	ATTATTTGCT	TTGGTTTCTG	TGACTGTGCT	CTAGGTGCAA
1801	AGACACATAT	TTTGTTCCCG	TTGCTCAGGC	TGGCCTCAAA	CTCTAAGGCT
4851	CCAGCAATCT	CTGGTCTCAG	CCAGAGACCT	GTAAAAATAG	ACACTTCAAA
4901	GATTATCATG	AATAAATA*			

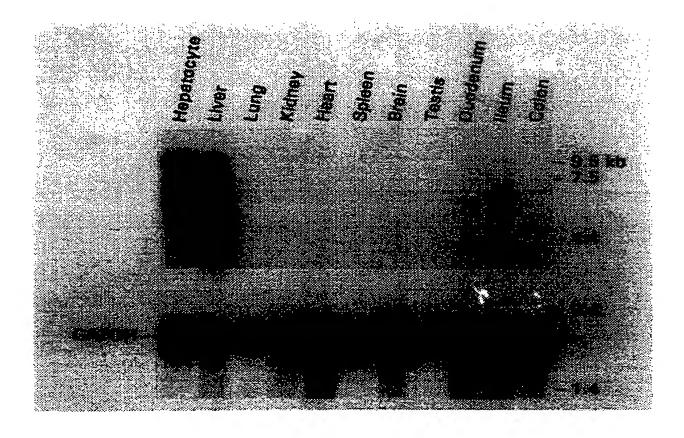


FIG. 2a

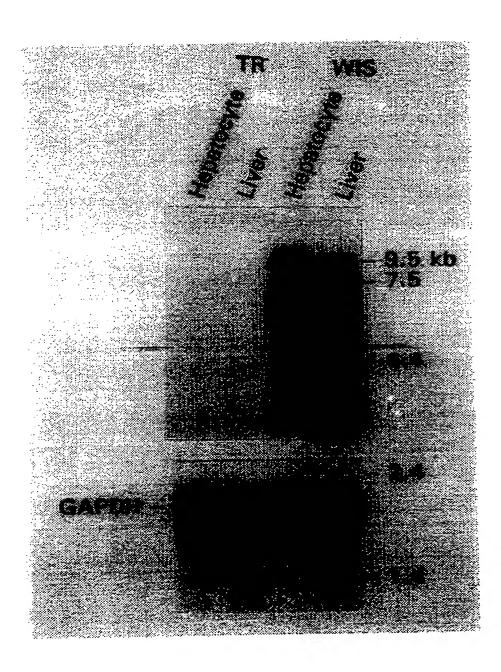


FIG. 2b

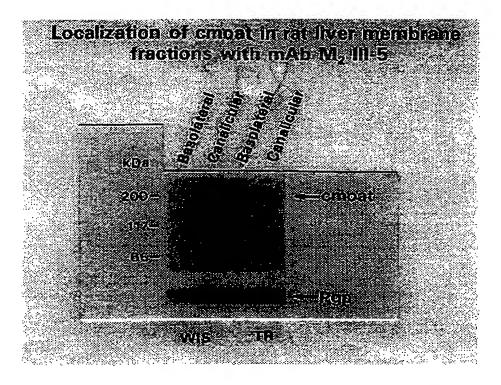
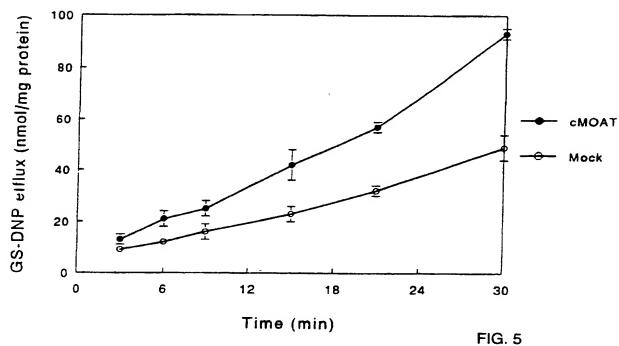


FIG. 3

1	MDKFCNSTFWDLSLLESPEADPPLCFEQT <u>VLVWIPLGFLWLLA</u> PWQLYSVYRSRTKRSS
61	TKFYLAKQVFVVFLLILAAIDLSLALTEDTGQATVPPVRYTNPILYLCTWLLVLAVQHS
121	QWCVRKNSW <u>FLSLFWILSVLCGVFQFQT</u> LIRALLKDSKSNMAYSYLFFVSYGFQIVLLII
181	TAFSGPSDSTQTPSVTASFLSSITFSWYDRTVLKGYKHPLTLEDVWDIDEGFKTRSVTSF
241	FEAAMTKDLQKARQAFQRRLQKSQRKPEATLHGLNKKQSQSQDVLVLEEAKKKSEKTTKI
301	YPKSWLIKSLFKTFHVVILKSFILKLIHDLLVFLNPQLLKLLIGFVKSSNSYVWFG <u>VIC</u>
361	* <u>ILMFAVTLIQSF</u> CLQSYFQHCFVLGMCVRTTVMSSIYKKALTLSNLARKQYTIGETVNLM
421	SVDSQKLMDATNYMQ <u>LVWSSVIQITLSIFFL</u> WRELGPS <u>ILAGVGVMVLLIPVNGVI</u> ATKI
481	RNIQVQNMKNKDKRLKIMNEILSGIKILKYFAWEPSFQEQVQGIRKKELKNLLRFGQLQS
541	LLIFILQITPILVSVVTFSVYVLVDSANVLNAEKAF1SITLFNILRFPLSMLPMVTSSII
601	QASVSVDRLERYLGGDDLDTSAIRAVSNFDKAVKFSEASFTWDPDLEAT <u>IODVNLDIKPG</u>
661	OLVAVVGTVGSGKBBLVSAMLGEMENVHGHITIOGSTAYVPOOSWIONGTIKDNILFGSE   : :.  .  .  :.:::    . rmrp1 GHVTLKGSVAYVPQQAWIQNDSLRENILFGRE
721	YNEKKYOOVLKACALLPDLEILPGGDMAEIGEKGINLSGGOKORVSLARAAYODADIYIL
	.   :.           :  :.     : LQEHCYKAYMEACALLPDLEILPSGDLTEIGEKGVITS TMRP1
781	LQEHCYKAYMEACALLPDLEILPSGDLTEIGEKGVITS rMRP1  DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS
781 841	LQEHCYKAYMEACALLPDLEILPSGDLTEIGEKGVITS IMRPI
	LQEHCYKAYMEACALLPDLEILPSGDLTEIGEKGVITS IMRPI DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS
841	LQEHCYKAYMEACALLPDLEILPSGDLTEIGEKGVITS IMRPI  DDPLSAVDAHVGKHIFNKUVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS  YRDLLDKKGVFARNWKTFMKHSGPEGEATVNNDSEAEDDDDGLIPTMEEIPEDAASLAMF
841 901	LQEHCYKAYMEACALLPDLEILPSGDLTEIGEKGVITS IMRPI  DDPLSAVDAHVGKHIFNKUVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS  YRDLLDKKGVFARNWKTFMKHSGPEGEATVNNDSEAEDDDDGLIPTMEEIPEDAASLAMF  RENSLRRTLSRSSRSSSRRGKSLKNSLKIKNVNVLKEKEKEVEGQKLIKKEFVETGKVKF
841 901 961	LQEHCYKAYMEACALLPDLEILPSGDLTEIGEKGVITS IMRPI  DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS  YRDLLDKKGVFARNWKTFMKHSGPEGEATVNNDSEAEDDDDGLIPTMEEIPEDAASLAMF  RENSLRRTLSRSSRSSSRRGKSLKNSLKIKNVNVLKEKEKEVEGQKLIKKEFVETGKVKF  SIYLKYLQAVGWWSILFIILFYGLNNVAFIGSNLWLSAWTSDSDNLNGTNNSSSHRDMRI
841 901 961 1021	DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS YRDLLDKKGVFARNWKTFMKHSGPEGEATVNNDSEAEDDDDGLIPTMEEIPEDAASLAMF RENSLRRTLSRSSRSSSRRGKSLKNSLKIKNVNVLKEKEKEVEGQKLIKKEFVETGKVKF SIYLKYLQAVGWWSILFIILFYGLNNVAFIGSNLWLSAWTSDSDNLNGTNNSSSHRDMRI GVFGALGLAQGICLLISTLWSIYAGRNASKALHGQLLTNILRAPMRFFDTTPTGRIVNRF
841 901 961 1021 1081	DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS YRDLLDKKGVFARNWKTFMKHSGPEGEATVNNDSEAEDDDDGLIPTMEEIPEDAASLAMF RENSLRRTLSRSSRSSSRRGKSLKNSLKIKNVNVLKEKEKEVEGQKLIKKEFVETGKVKF SIYLKYLQAVGWWSILFIILFYGLNNVAFIGSNLWLSAWTSDSDNLNGTNNSSSHRDMRI GVFGALGLAQGICLLISTLWSIYAGRNASKALHGQLLTNILRAPMRFFDTTPTGRIVNRF SGDISTVDDLLPQTLRSWMMCFFGIAGTLVMICMATPVFAIIIIPLSILYISVOVFYVAT
841 901 961 1021 1081 1141	DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS YRDLLDKKGVFARNWKTFMKHSGPEGEATVNNDSEAEDDDDGLIPTMEEIPEDAASLAMF RENSLRRTLSRSSRSSSRRGKSLKNSLKIKNVNVLKEKEKEVEGQKLIKKEFVETGKVKF SIYLKYLQAVGWWSILFIILFYGLNNVAFIGSNLWLSAWTSDSDNLNGTNNSSSHRDMRI GVFGALGLAQGICLLISTLWSIYAGRNASKALHGQLLTNILRAPMRFFDTTPTGRIVNRF SGDISTVDDLLPQTLRSWMMCFFGIAGTLVMICMATPVFAIIIIPLSILYISVQVFYVAT
841 901 961 1021 1081 1141 1201	DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS YRDLLDKKGVFARNWKTFMKHSGPEGEATVNNDSEAEDDDDGLIPTMEEIPEDAASLAMF RENSLRRTLSRSSRSSSRRGKSLKNSLKIKNVNVLKEKEKEVEGQKLIKKEFVETGKVKF SIYLKYLQAVGWWSILFIILFYGLNNVAFIGSNLWLSAWTSDSDNLNGTNNSSSHRDMRI GVFGALGLAQGICLLISTLWSIYACRNASKALHGQLLTNILRAPMRFFDTTPTGRIVNRF SGDISTVDDLLPQTLRSWMMCFFGIAGTLVMICMATPVFAIIIIPLSILYISVOVFYVAT SRQLRRLDSVTKSPIYSHFSETVTGLPIIRAFEHQQRFLAWNEKQIDINGKCVFSWITSN RWLAIRLELVGNLVVFCSALLLVIYRKTUTGDVVGFVLSNALNITQTLNWLVRMTSEAET
841 901 961 1021 1081 1141 1201 1261	DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS YRDLLDKKGVFARNWKTFMKHSGPEGEATVNNDSEAEDDDDGLIPTMEEIPEDAASLAMF RENSLRRTLSRSSRSSSRRGKSLKNSLKIKNVNVLKEKEKEVEGQKLIKKEFVETGKVKF SIYLKYLQAVGWWSILFIILFYGLNNVAFIGSNLWLSAWTSDSDNLNGTNNSSSHRDMRI GVFGALGLAQGICLLISTLWSIYACRNASKALHGQLLTNILRAPMRFFDTTPTGRIVNRF SGDISTVDDLLPQTLRSWMMCFFGIAGTLVMICMATPVFAIIIIPLSILYISVOVFYVAT SRQLRRLDSVTKSPIYSHFSETVTGLPIIRAFEHQQRFLAWNEKQIDINGKCVFSWITSN RWLAIRLELVGNLVVFCSALLLVIYRKTUTGDVVGFVLSNALNITQTLNWLVRMTSEAET NIVAVERISEYINVENEAPWVTDKRPPADWPRHGEIQFNNYQVRYRPELDLVLKGITCNI
841 901 961 1021 1081 1141 1201 1261 1321	DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS YRDLLDKKGVFARNWKTFMKHSGPEGEATVNNDSEAEDDDDGLIPTMEEIPEDAASLAME RENSLRRTLSRSSRSSSRRGKSLKNSLKIKNVNVLKEKEKEVEGQKLIKKEFVETGKVKF SIYLKYLQAVGWWSILFIILFYGLNNVAFIGSNLWLSAWTSDSDNLNGTNNSSSHRDMRI GVFGALGLAQGICLLISTLWSIYAGRNASKALHGQLLTNILRAPMRFFDTTPTGRIVNRF SGDISTVDDLLPQTLRSWMMCFFGIAGTLVMICMATPVFAIIIPLSILYISVOVFYVAT SRQLRRLDSVTKSPIYSHFSETVTGLPIIRAFEHQQRFLAWNEKQIDINGKCVFSWITSN RWLAIRLELVGNLVVFCSALLLVIYRKTDTGDVVGFVLSNALNITQTLNWLVRMTSEAET NIVAVERISEYINVENEAPWVTDKRPPADWPRHGEIQFNNYQVRYRPELDLVLKGITCNI KSGEKVGVVGRTGAGKBBLTNCLFRILESAGGOIIIDGIDVASIGLHDLRERLTIIPODI

# GS-DNP transport in COS-7 cells transfected with cMOAT/'Mock'; T= 15°C



BNSDOCID: <WO\_\_\_\_ \_\_\_\_9731111A2\_i\_>

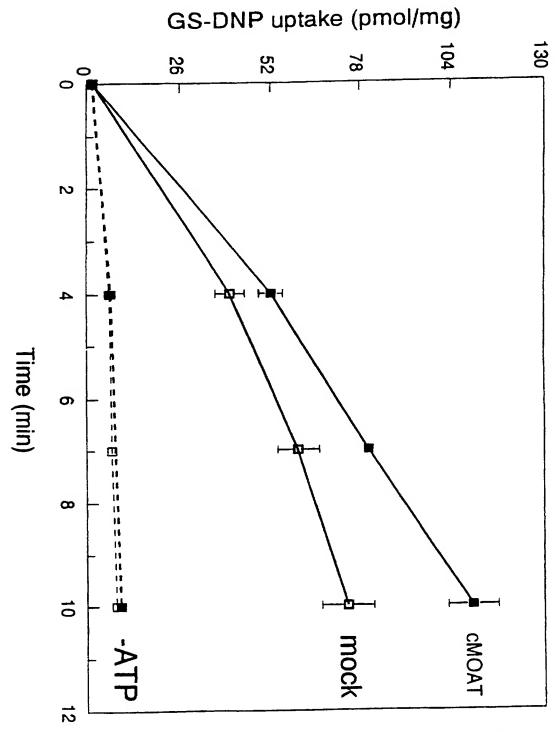


FIG. 6

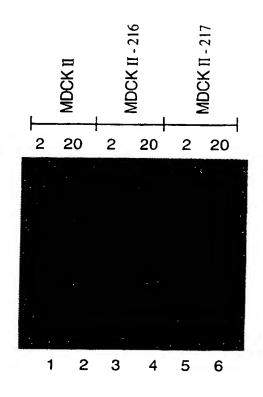


FIG. 7



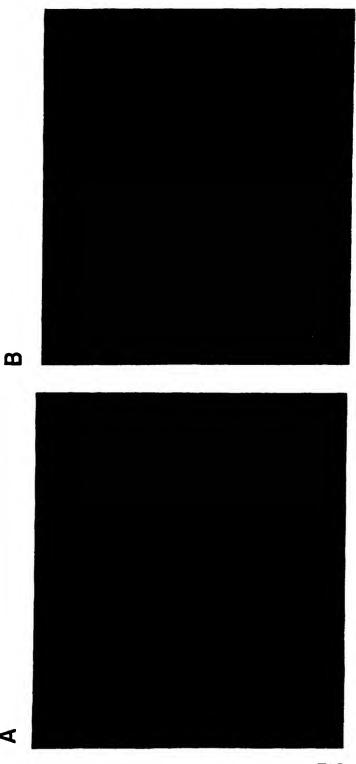
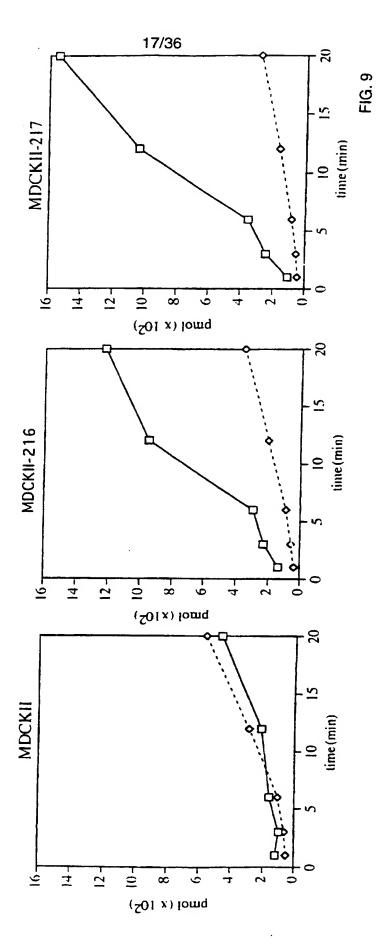


FIG. 8



18/36

1 MLEKFONSTF WASSFLOSPE ADLPLOFEOT VLVWIPLGFL VLLAPHOLLH VYKSRTKRSS TIKLYLAKOV FVGFLLILAA IELALVLTED SGQATVPAVR 101 YTHPSLYLGT WLLVLLIOYS ROWCVOKHSW FLSLFWILST CCGTFOFOTL IRTLLGCOMS HLAYSCLFFT SYGFOILILT FSAFSEHNES SHIPSSTASF 201 LSSITYSWYD SIILKGYKRP LILEDVWEVD EEMKTKILYS KFETHMKREL QKARRALQRR QEKSSQONSG ARLPGLNKNO SQSQDALVLE DVEKKKKKSG 301 TKKDYPKSWL NKALFKTFYM VLLKSFLLKL <u>VND1FTFVSP QL</u>LKLLISFA SDRDTYLWIG <u>YLCAILLFTA ALIOSFCLO</u>C YFQLCFKLGV KVRTAIMASV 401 YKKALTISNI ARKEYTYGET VNIMSVDAGK IMOVTNEMH<u>N LUSSVLOTVI STEFLU</u>RELG PS<u>VLAGVGVM VLVTPINATI</u> STKSKTTQVK NMKNKDKRIK 501 IMMEILSGIK ILKYFAWEPS FROOVONLRK KELKNLLAFS QLQCVVIFVF QLTPVLVSVV TFSVVVLVOS MNILDAQKAF TSITLFNILR FPLSMLPMMI SSMLOASVST ERLEKYLGGD DEDTSAIRHD CHFDKAMOFS EASFTWEHDS EATVRDVNED IMAGGEVAVI GPVGSGKSSE ISAMEGEMEN VHGHETIKGT TAYVPOOSWI ONGTIXDNIL FGTEFNEKRY OOVLEACALL POLENLPGGD LAEIGEKGIN LSGGOKORIS LARATYONLD IYLLDDPLSA VDAHVGKHIF NKVLGPNGLL KGKTRLLVTH SMHFLPOVDE IVVLGNGTIV EKGSYSALLA KKGEFAKNLK TFLRHTGPEE EATVHOGSEE EDDDYGLISS VEEIPEDAAS 801 ITMRRENSFR RTLSRSSRSN GRHLKSLRNS LKTRNVNSLK EDEELVKGOK LIKKEFIETG KVKFSIYLEY LOAIGLESIF FIILAFVMNS VAFIGSNLWL SAWTSDSKIF HSTDYPASOR DHRVGVYGAL GLAGGIFVFI AHFWSAFGFV HASHILHKOL LHNILRAPHR FFDTTPTGRI VNRFAGDIST VDDTLPOSLR 1001 SWITCFLOII STLVMICMAT PUFTIIVIPL GIIYVSVOMF TVSTSROLRR LDSVTRSPIY SHFSETVSGL PVIRAFEHOO RFLKHNEERI DTNOKCVFSW ITSMRWLAIR LELVGHLTVF FSALMHVIYR DILSGDTVCF VLSHALNITO ILMULVRMIS EIETNIVAVE RITEYIKVEN EAPWYDKRP PPDWPSKGKI 1301 OFHNYQVRYR PELDLYLRGI TCDIGSHEKI GVVGRTGAGK SSLINCLFRI LEAAGGOIII DGVDIASIGL HDLREKLTII PODPILFSGS LRMNLDPFNN 1401 YSDEEJWKAL ELAHLKSFVA SLOLGLSHEV TEAGGH<u>LSIG OROLLCL</u>GRA LLRKSK<u>ILVL DE</u>ATAAVDLE TONLIGTTIG NEFAHCTVIT JAHRLHTIMD 1501 SOKYMYLONG KITEYGSPEE LLOTPGPFYF MAKEAGTENV WSTKF\*

FIG. 10

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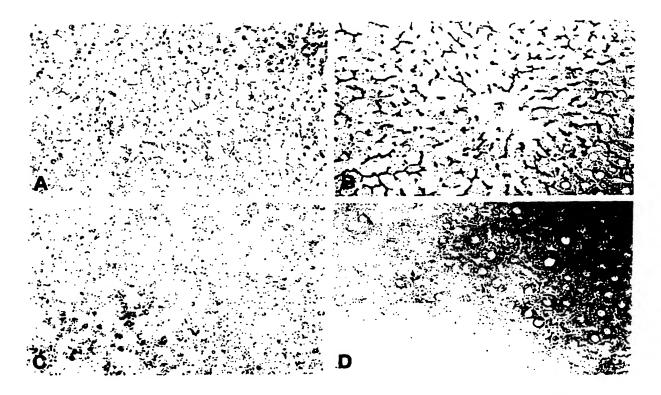
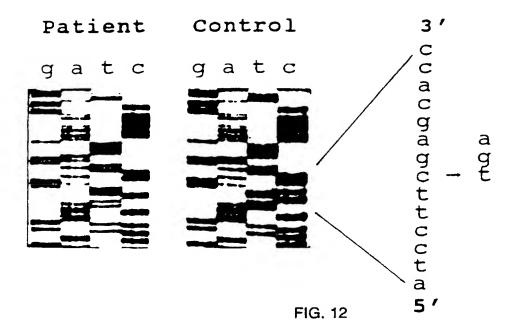


FIG. 11



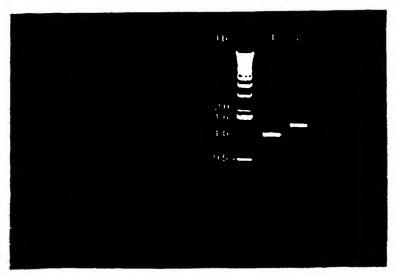
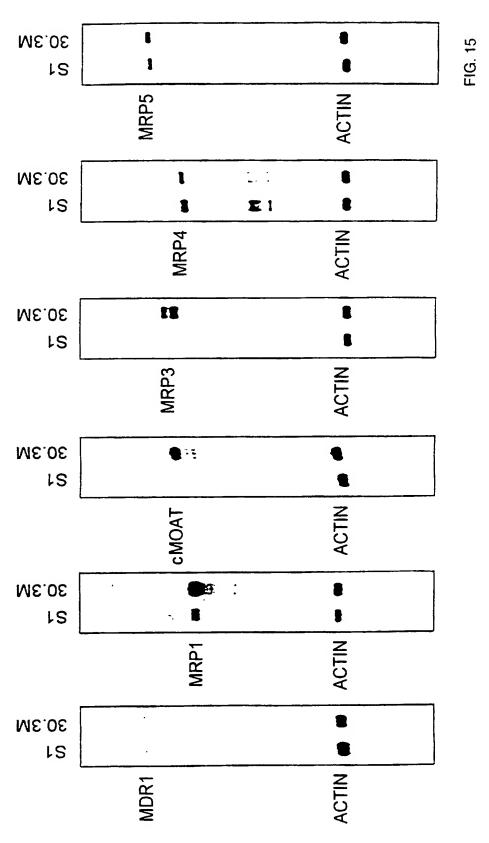
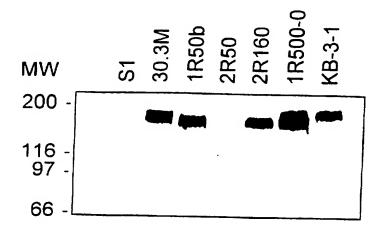


FIG. 13

ä

cMOAT/	MRP1 MRP2 MRP3 MRP4 MRP5 HSUR	FVLRHINVTI LVLRGITCDI LVLRDLSLHV TI	NGGEKVGIVG GSMEKIGVVG HGGEKVGIVG  KPKEKIGIVG SPGQKIGICG	RTGAGKSSLT RTGAGKSSLT RTGAGKSSMT  RTGSGKSSLG RTGSGKSSLG	LGLFRINESA NCLFRILEAA LACSRILEAA  MALFRIVELS LAFFRWOTF	EGELIIDGIN GGQIIIDGVD KGEIRIDGLN  GGCIKIDGVR EGHIIIDGID
CMOAT/	MRP1 MRP2 MRP3 MRP4 MRP5 HSUR	IAKIGLHDLR IASIGLHDLR VADIGFHDVR  ISDIGLADLR IAKLPLHTLR	FKITIIPQDP EKLTIIPQDP CQMTIIPRDP SKLSIIPQEP SRLSIILQDP	VLFSGSLRMN ILFSGSLRMN ILFSGTLRMN ILFSGTLRMN VLFSGTTRFN	LDPFSQYSDE LDPFGNNYSDE LDPFGSYSEE LDPFGSYSEE	EVWTSLELAH BIWKALELAH DIWWALELSH OTHORALERTH TLWEALEIAQ
CMOAT/	MRP1 MRP3 MRP4 MRP5 HSUR	* LKDFVSALPD LKSFVASLQL LHTFVSSQPA LKETIEDLPG MKECIAQLPL LKLVVKALPG	KLDHECAEGG GLSHEVTEAG GLDFQCSEGG KMDTELAESG KLESEVMENG	* ******* ENLSVGGRGL GNLSIGGRGL ENLSVGGRGL SNSSVGGRGL DNPSVGBRGL ENPSOGGRGL	********* VCLARALLRK LCLGRALLRK VCLARALLRK VCLARALLRK LCIARALLRK	** **** TKILVLDBAT SKILVLDBAT SRILVLDBAT NQILIIDBAT CKILILDBAT TSIFIMDBAT
CMOAT/	MRP1 MRP2 MRP3 MRP4 MRP5 HSUR	AAVDLETDDL AAVDLETDNL AAIDLETDNL AANDPRTDEL AAMDTETDLL ASIDMATENI	1QSTIRTQFE IQTTIQNEFA IQTTIRTQFD IQKIREKFA IQETIREAFA LQKVVMTAFA	******** DCTVLTIAHR HCTVITIAHR TCTVLTIAHR HCTVLTIAHR DCTWLTIAHR	LNTIMDSDKV LNTIMDSDKV LNTIMDYTRV LNTIDSFKI LHTVLGSDRI	IVLDKGEIQE MVLDNGKIIE LVLDKGVVAE MVLDSGRLKE MVLAQGQVVE IVLKRGAILE
CMOAT/	MRP1 MRP2 MRP3 MRP4 MRP5	YGAPSDLLQQ YGSPEELLQI FDSPANLIAA YDEPYVLLQN FDTPSVLLSN FDKPEKLLSN	R. GLFYSMAK P. GPFYFMAK R. GIFYGMAR KESLFYKWVQ DSSRFYAMFA	** DAGL EAGI DAGL QLG. AAE.		FIG. 14





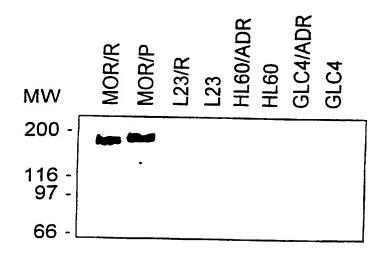
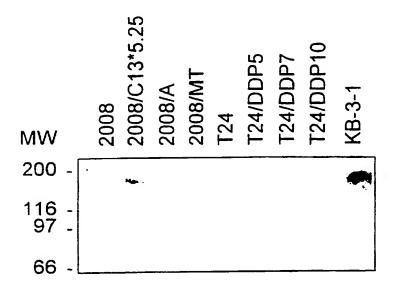


FIG. 16a



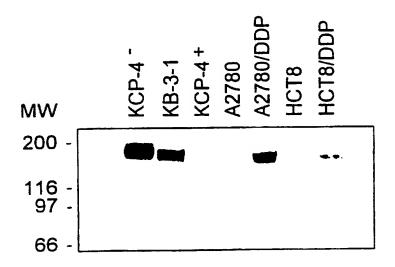


FIG. 16b

mrp3.sed	Length: 4762 February 18, 1997 11:02 Type: N Check: 1908
1	CTCCGGCGCC CGCTCTGCCC GCCGcTGGgT CCGACCGCGc TcGCCTTCCT
51	TGCAGCeGCG CeTCGGCCCC ATGGACGCCC TGTGCGGTTC CGGGGAGeTC
101	GGCTCCAAgt tCTGGGACTC CAACCTGTCT GTGCACACAG AAAACCCGGA
151	CCTCACTCCC TGCTTCCAGA ACTCCCTGCT GGCCTGGGTG CCCTGCATcT
201	ACCTGTGGGT CGCCCTGCCC TGCTACTTGC TcTACcTGCG GCACCATTGT
251	CGTGGCTACA TCATCCTCTC CCACCTGTCC AAGCTCAAGA TGGTCCTGGG
301	TGTCCTGCTG TGnnnngtgt gctctcctgg cgaccttttt tactcttcca
351	tggcctggtc catggccgcc cctgcccctg ttttctttgt cacccccttg
401	gtggtggggg tcaccatgCT GCTGGCCACC CTGCTGATAC AGTATGAsCG
451	GCTGCAGGGC GTACAGTCTT CGGGGGTCCT CATTATCTTC TGGTTCCTGT
501	GTGTGGTCTG CGCCATCGTC CCATTCCGCT CCAAGATCCT TTTAGCCAAG
551	GCAGAGGGTG AGATCTCAGA CCCCTTCCGC TTCACCACCT TCTACATCCA
601	CTTTGCCCTG GTACTCTCTG CCCTCATCTT GGCCTGCTTC AGGGAGAAAC
651	CTCCATTTTT CTCCGCAAAG AATGTCGACC CTAACCCCTA CCCTGAGACC
701	AGCGCTGGCT TTCTCTCCCG CCTGTTTTTC TGGTGGTTCA CAAAGATGGC
751	CATCTATGGC TACCGGCATC CCCTGGAGGA GAAGGACCTC TGGTCCCTAA
801	AGGAAGAGGA CAGATCCCAG ATGGTGGTGC AGCAGCTGCT GGAGGCATGG
851	AGGAAGCAGG AAAAGCAGAC GGCACGACAC AAGGCTTCAG CAGCACCTGG
901	GAAAAATGCC TCCGGCGAGG ACGAGGTGCT GCTGGGTGCC CGGCCCAGGC
951	CCCGGAAGCC CTCCTTCCTG AAGGCCCTGC TGGCCACCTT CGGCTCCAGC
1001	TTCCTCATCA GTGCCTGCTT CAAGCTTATC CAGGACCTGC TCTCCTTCAT
1051	CAATCCACAG CTGCTCAGCA TCCTGATCAG GTTTATCTCC AACCCCATGG
1101	CCCCCTCCTG GTGGGGCTTC CTGGTGGCTG GGCTTGATGT TCCTGTTGCT
1151	CCATGGATGC AGTCGCTGAT CTTACAACAC TATTACCACT ACATCTTTGT
1201	GACTGGGGTG AAGTTTCGTA CTGGGATCAT GGGTGTCATC TACAGGAAGG
1251	CTCTGGTTAT CACCAACTCA GTCAAACGTG CGTCCACTGT GGGGGAAATT

FIG. 17a

1301	GTCAACCTCA	TGTCAGTGGA	IGCCCAGCGC	TICATGGACC	11000001
1351	CCTCAATCTG	CTGTGGTCAG	CACCCCTGCA	GATCATCCTG	GCGATCTACT
1401	TCCTCTGGCA	GAACCTAGGT	CCCTCTGTCC	TGGCTGGAGT	CGCTTTCATC
1451	GTCTTGCTGA	TTCCACTCAA	CGGAGCTGTG	GCCGTGAAGA	TGCGCGCCTT
1501	CCAGGTAAAG	CAAATGAAAT	TGAAGGACTC	GCGCATCAAG	CTGATGAGTG
1551	AGATCCTGAA	CGGCATCAAG	GTGCTGAAGC	TGTACGCCTG	GGAGCCCAGC
1601	TTCCTGAAGC	AGGTGGAGGG	CATCAGGCAG	GGTGAGCTCC	AGCTGCTGCG
1651	CACGGCGGCC	TACCTCCACA	CCACAACCAC	CTTCACCTGG	ATGTGCAGCC
1701	CCTTCCTGGT	GACCCTGATC	ACCCTCTGGG	TGTACGTGTA	CGTGGACCCA
1751	AACAATGTGC	TGGACGCCGA	GAAGGCCTTT	GTGTCTGTGT	CCTTGTTTAA
1801	TATCTTAAGA	CTTCCCCTCA	ACATGCTGCC	CCAGTTAATC	AGCAACCTGA
1851	CTCAGGCCAG	TGTGTCTCTG	AAACGGATCC	AGCAATTCCT	GAGCCAAGAG
1901	GAACTTGACC	CCCAGAGTGT	GGAAAGAAAG	ACCATCTCCC	CAGGCTATGC
1951	CATCACCATA	CACAGTGGCA	CCTTCACCTG	GGCCCAGGAC	CTGCCCCCA
2001	CTCTGCACAG	CCTAGACATC	CAGGTCCCGA	AAGGGGCACT	GGTGGCCGTG
2051	GTGGGGCCTG	TGGGCTGTGG	GAAGTCCTCC	CTGGTGTCTG	CCCTGCTGGG
2101	AGAGATGGAG	AAGCTAGAAG	GCAAAGTGCA	CATGAAGGGC	TCCGTGGCCT
2151	ATGTGCCCCA	GCAGGCATGG	ATCCAGAACT	GCACTCTTCA	GGAAAACGTG
2201	CTTTTCGGCA	AAGCCCTGAA	CCCCAAGCGC	TACCAGCAGA	CTCTGGAGGC
2251	CTGTGCCTTG	CTAGCTGACC	TGGAGATGCT	GCCTGGTGGG	GATCAGACAG
2301	agattggaga	gAAGGGCATT	AACCTGTCTG	GGGGCCAGCG	GCAGCGbGTC
2351	AGTCTGGCTC	GAGCTGTTTA	CAGTGATGCC	Gatattttct	TGCTGGATGA
2401	CCCACTGTCC	GCGGTGGACT	CTCATGTGGC	CAAGCACATC	TTTGACCACG
2451	TCATCGGGCC	AGAAGGCGTG	CTGGCAGGCA	AGACGCGAGT	GCTGGTGACG
2501	CACGGCATTA	GCTTCCTGCC	CCAGACAGAC	TTCATCATTG	TGCTAGCTGA
2551	TGGACAGGTG	TCTGAGATGG	GCCCGTACCC	AGCCCTGCTG	CAGCGCAACG
2601	GCTCCTTTGC	CAACTTTCTC	TGCAACTATG	CCCCGATGA	GGACCAAGGG
2651	CACCTGGAGG	ACAGCTGGAC	CGCGTTGGAA	GGTGCAGAGG	ATAAGGAGGC
2701	ACTGCTGATT	GAAGACACAC	TCAGCAACCA	CACGGATCTG	ACAGACAATG
2751	ATCCAGTCAC	CTATGTGGTC	CAGAAGCAGT	TTATGAGACA	GCTGAGTGCC
2801	CTGTCCTCAG	ATGGGGAGGG	ACAGGGTCGG	CCTGTACCCC	GGAGGCACCT

27/36

2851 GGGTCCATCA GAGAAGGTGC AGGTGACAGA GGCGAAGGCA GATGGGGCAC 2901 TGACCCAGGA GGAGAAAGCA GCCATTGGCA CTGTGGAGCT CAGTGTGTTC 2951 TGGGATTATG CCAAGGCCGT GGGGCTCTGT ACCACGCTGG CCATCTGTCT 3001 CCTGTATGTG GGTCAAAGTG CGGCTGCCAT TGGAGCCAAT GTGTGGCTCA 3051 GTGCCTGGAC AAATGATGCC ATGGCAGACA GTAGACAGAA CAACACTTCC 3101 CTGAGGCTGG GCGTCTATGC TGCTTTAGGA ATTCTGCAAG GGTTCTTGGT 3151 GATGCTGGCA GCCATGGCCA TGGCAGCGGG TGGCATCCAG GCTGCCCGTG 3201 TGTTGCACCA GGCACTGCTG CACAACAAGA TACGCTCGCC ACAGTCCTTC 3251 TTTGACACCA CACCATCAGG CCGCATCCTG AACTGCTTCT CCAAGGACAT 3301 CTATGTCGTT GATGAGGTTC TGGCCCCTGT CATCCTCATG CTGCTCAATT 3351 CCTTCTTCAA CGCCATCTCC ACTCTTGTGG TCATCATGGC CAGCACGCCG 3401 CTCTTCACTG TGGTCATCCT GCCCCTGGCT GTGCTCTACA CCTTAGTGCA 3451 GCGCTtCTAT GCAGCCACAT CACGGCAACT GAAGCGGCTG GAATCAGTCA 3501 GCCGCTCACC TATCTACTCC CACTTTTCGG AGACAGTGAC TGGTGCCAGT 3551 GTCATCCGGG CCTACAACCG CAGCCGGGAT TTTGAGATCA TCAGTGATAC 3601 TAAGGTGGAT GCCAACCAGA GAAGCTGCTA CCCCTACATC ATCTCCAACC 3651 GGTGGCTGAG CATCGGAGTG GAGTTCGTGG GGAACTGCGT GGTGCTCTTT 3701 GCTGCACTAT TTGCCGTCAT CGGGAGGAGC AGCCTGAACC CGGGGCTGGT 3751 GGGCCTTTCT GTGTCCTACT CCTTGCAGGT GACATTTGCT CTGAACTGGA 3801 TGATACGAAT GATGTCAGAT TTGGAATCTA ACATCGTGGC TGTGGAGAGG 3851 GTCAAGGAGT ACTCCAAGAC AGAGACAGAG GCGCCCTGGG TGGTGGAAGG 3901 CAGCCGCCCT CCCGAAGGTT GGCCCCCACG TGGGGAGGTG GAGTTCCGGA 3951 ATTATTCTGT GCGCTACCGG CCGGGCCTAG ACCTGGTGCT GAGAGACCTG 4001 AGTCTGCATG TGCATGGTGG CGAGAAGGTG GGGATCGTGG GCCGCACTGG 4051 GGCTGGCAAG TCTTCCATGA CCCLTTGCCT GTTCCGCATc CTGGAGGCGG 4101 CAAAGGGTGA AATCCGCATT GATGGCCTCA ATGTGGCAGA CATCGGCCTC 4151 CATGACCTGC GCTCTCAGCT GACCATCATC CCGCaGGACC CCATCCTGTT 4201 CTCGGGGACC CTGCGCATGA ACCTGGACCC CTTCGGCAGC TACTCAGAGG 4251 AGGACATTTG GTGGGCTTTG GAGCTGTCCC ACCTGCACAC GTTTGTgAGC 4301 TCCCAGCCGG CAGGCCTGGA CTTCCAGTGC TCAGAGGGCG GGGAGAATCT

1351	CAGCGTGGGC	CAGAGGCAGC	TCGTGTGCCT	GGCCCGAGCC	CTGCTCCGCA
1401	AGAGCCGCAT	CCTGGTTTTA	GACGAGGCCA	CAGCTGCCAT	CGACCTGGAG
451	ACTGACAACC	TCATCCAGGC	TACCATCCGC	ACCCAGTTTG	ATACCTGCAC
501	TGTCCTGACC	ATCGCACACC	GGCTTAACAC	TATCATGGAC	TACACCAGGG
551	TCCTGGTCCT	GGACAAAGGA	GTAGTAGCTG	AGTTTGATTC	TCCAGCCAAC
601	CTCATTGCAG	CTAGAGGCAT	CTTCTACGGG	ATGGCCAGAG	ATGCTGGACT
651	TGCCTAAAAT	ATATTCCTGA	GATTTCCTCC	TGGCCTTTCC	TGGTTTTCAT
701	CAGGAAGGAA	ATGACACCAA	ATATGTCCGC	AGAATGGACT	TGCCGGATTC
751	CGGCCGGAAT	TC			

mrp3.pep	Length: 1529 February 18, 1997 11:03 Type: P Check: 8806
1	MDALCGSGEL GSKFWDSNLS VHTENPDLTP CFQNSLLAWV PCIYLWVALP
51	CYLLYLRHHC RGYIILSHLS KLKMVLGVLL XXVCSPGDLF YSSMAWSMAA
101	PAPVFFVTPL VVGVTMLLAT LLIQYXRLQG VQSSGVLIIF WFLCVVCAIV
151	PFRSKILLAK AEGEISDPFR FTTFYIHFAL VLSALILACF REKPPFFSAK
201	NVDPNPYPET SAGFLSRLFF WWFTKMAIYG YRHPLEEKDL WSLKEEDRSQ
251	MVVQQLLEAW RKQEKQTARH KASAAPGKNA SGEDEVLLGA RPRPRKPSFL
301	KALLATFGSS FLISACFKLI QDLLSFINPQ LLSILIRFIS NPMAPSWWGF
351	LVAGLDVPVA PWMQSLILQH YYHYIFVTGV KFRTGIMGVI YRKALVITNS
401	VKRASTVGEI VNLMSVDAQR FMDLAPFLNL LWSAPLQIIL AIYFLWQNLG
451	PSVLAGVAFM VLLIPLNGAV AVKMRAFQVK QMKLKDSRIK LMSEILNGIK
. 501	VLKLYAWEPS FLKQVEGIRQ GELQLLRTAA YLHTTTTFTW MCSPFLVTLI
551	TLWVYVYVDP NNVLDAEKAF VSVSLFNILR LPLNMLPQLI SNLTQASVSL
601	KRIQQFLSQE ELDPQSVERK TISPGYAITI HSGTFTWAQD LPPTLHSLDI
651	QVPKGALVAV VGPVGCGKSS LVSALLGEME KLEGKVHMKG SVAYVPQQAW
701	IQNCTLQENV LFGKALNPKR YQQTLEACAL LADLEMLPGG DQTEIGEKGI
751	NLSGGQRQRV SLARAVYSDA DIFLLDDPLS AVDSHVAKHI FDHVIGPEGV
801	LAGKTRVLVT HGISFLPQTD FIIVLADGQV SEMGPYPALL QRNGSFANFL
851	CNYAPDEDQG HLEDSWTALE GAEDKEALLI EDTLSNHTDL TDNDPVTYVV
901	QKQFMRQLSA LSSDGEGQGR PVPRRHLGPS EKVQVTEAKA DGALTQEEKA
951	AIGTVELSVF WDYAKAVGLC TTLAICLLYV GQSAAAIGAN VWLSAWTNDA
1001	MADSRQNNTS LRLGVYAALG ILQGFLVMLA AMAMAAGGIQ AARVLHQALL
1051	HNKIRSPOSF FOTTPSGRIL NCFSKDIYVV DEVLAPVILM LLNSFFNAIS
1101	
1151	
1201	EFVGNCVVLF AALFAVIGRS SLNPGLVGLS VSYSLQVTFA LNWMIRMMSD
1251	LESNIVAVER VKEYSKTETE APWVVEGSRP PEGWPPRGEV EFRNYSVRYR

FIG. 17b

1301	PGLDLVLRDL	SLHVHGGEKV	GIVGRTGAGK	SSMTLCLFRI	LEAAKGEIR
1351	DGLNVADIGL	HDLRSQLTII	PQDPILFSGT	LRMNLDPFGS	YSEEDIWWAI
1401	ELSHLHTFVS	SQPAGLDFQC	SEGGENLSVG	QRQLVCLARA	LLRKSRILVI
1451	DEATAAIDLE	TONLIQATIR	TQFDTCTVLT	IAHRLNTIMD	YTRVLVLDKO
1501	VVAEFDSPAN	LIAARGIFYG	MARDAGLA*		

- mrp4.seq Length: 486 September 26, 1996 09:58 Type: N Check: 879 ..
- 1 acttaaagaa accattgaag atcttcctgg taaaatggat actgaattag
- 51 cagaatcagg atccaatttt agtgttggac aaagacaact ggtgtgcctt
- 101 gccagggcaa ttctcaggaa aaatcagata ttgattattg atgaagcgac
- 151 ggcaaatgtg gatccaagaa ctgatgagtt aatacaaaaa aaaatccggg
- 201 agaaatttgc ccactgcacc gtgctaacca ttgcacacag attgaacacc
- 251 attattgaca gcgacaagat aatggtttta gattcaggaa gactgaaaga
- 301 atatgatgag ccgtatgttt tgctgcaaaa taaagagagc ctattttaca
- 351 agatggtgca acaacttggc aaggcagaag nnnnnnnct cactgaaaca
- 401 gcaaaacngg tatacttcaa aagaaattnt ncacatattg gtgacnctgn
  - 451 ccacatggtt acaaacngtt ncaatggncn nnnntc

FIG. 18a

- mrp5a.con Length: 1761 September 10, 1996 11:56 Type: N Check: 5118 ..
- 1 CATTGCAATC AGTGGAACCT TCGCTTATGT GGCCCAGCAG GCTGGATCCT
- 51 CAATGCTACT CTGAGAGACA ACATCCTGTT TGGGAAGGAA TATGATGAAG
- 101 AAAGATACAA CTCTGTGCTG AACAGCTGCT GCCTGAGGCC
  TGACCTGGCC
- 151 ATTCTTCCCA GCAGCGACCT GACGGAGATT GGAGAGCGAG
  GAGCCAACCT
- 201 GAGCGGTGGG CAGCGCCAGA GGATCAGCCT TGCCCGGGCC
  TTGTATAGTG
- 251 ACAGGAGCAT CTACATCCTG GACGACCCCC TCAGTGCCTT AGATGCCCAT
- 301 GTGGGCAACC ACATCTTCAA TAGTGCTATC CGGAAACATC TCAAGTCCAA
- 351 GACAGTTCTG TTTnnnGTTA CCCACCAGTT ACAGTACCTG
  GTTGACTGTG
- 401 ATGAAGTGAT CTTCATGAAA GAGGGCTGTA TTACGGAAAG AGGCACCCAT
- 451 GAGGAACTGA TGAATTTAAA TGGTGACTAT GCTACCATTT
  TTAATAACCT
- 501 GTTGCTGGGA GAGACACCGC CAGTTGAGAT CAATTCAAAA AAGGAAACCA
- 551 GTGGTTCACA GAAGAAGTCA CAAGACAAGG GTCCTAAAAC AGGATCAGTA
- 601 AAGAAGGAAA AAGCAGTAAA GCCAGAGGAA GGGCAGCTTG
- 651 AGAGAAAGGG CAGGGTTCAG TGCCCTGGTC AGTATATGGT GTCTACATCC
- 701 AGGCTGCTGG GGGCCCCTTG GCATTTCCTG GTTATTATGG
- 751 GGCCTGAATG TAGGCAGCAC CGCCTTCAGC ACCTGGTGGT TGAGTTACTG
- 801 GATCAAGCAA GGAAGCGGGA ACACCACTGT GACTCGAGGG
  AACGAGACCT
  FIG. 19a

- 851 CGGTGAGTGA CAGCATGAAG GACAATCCTC ATATGCAGTA CTATGCCAGC
- 901 ATCTACGCCC TCTCCATGGC AGTCATGCTG ATCCTGAAAG CCATTCGAGG
- 951 AGTTGTCTTT GTCAAGGGCA CGCTGCGAGC TTCCTCCCGG
- 1001 AGCTTTTCCG AAGGATCCTT CGAAGCCCTA TGAAGTTTTT TGACACGACC
- 1051 CCCACAGGGA GGATTCTCAA CAGGTTTTCC AAAGACATGG ATGAAGTTGA
- 1101 CGTGCGGCTG CCGTTCCAGG CCGAGATGTT CATCCAGAAC GTTATCCTGG
- 1151 TGTTCTTCTG TGTGGGAATG ATCGCAGGAG TCTTCCCGTG
- 1201 GCAGTGGGGC CCCTTGTCAT CCTCTTTTCA GTCCTGCACA
- 1251 GGTCCTGATT CGGGAGCTGA AGCGTCTGGA CAATATCACG CAGTCACCTT
- 1301 TCCTCTCCCA CATCACGTCC AGCATACAGG GCCTTGCCAC CATCCACGCC
- 1351 TACAATAAAG GGCAGGAGTT TCTGCACAGA TACCAGGAGC TGCTGGATGA
- 1401 CAACCAAGCT CCTTTTTTT TGTTTACGTG TGCGATGCGG TGGCTGGCTG
- 1451 TGCGGCTGGA CCTCATCAGC ATCGCCCTCA TCACCACCAC GGGGCTGATG
- 1501 ATCGTTCTTA TGCACGGGCA GATTCCCCCA GCCTATGCGG GTCTCGCCAT
- 1551 CTCTTATGCT GTCCAGTTAA CGGGGCTGTT CCAGTTTACG GTCAGACTGG
- 1601 CATCTGAGAC AGAAGCTCGA TTCACCTCGG TGGAGAGGAT CAATCACTAC
- 1651 ATTAAGACTC TGTCCTTGGA AGCACCTGCC AGAATTAAGA ACAAGGCTCC
- 1701 CTCCCCTGAC TGGCCCCAGG AGGGAGAGGA agAaGGTGAa CsTTTtGAGA
  - 1751 AAGGGGCCTT T

#### 34/36

- mrp5b.con Length: 2167 November 19, 1996 14:33 Type: N Check: 6982 ...
- 1 TTATGACCmc tGrAnTrrCg CCmAGGGGGS AgAkkrACCn TTcaTkAgAA
- 51 GGaaCArAaG aTGgGTACsG AgAAaCtTCc ttTtTTaTCc tAaAagaaAg
- 101 TATYCTTCA CGATCAAACC TAAAGAGAAG ATTGGCATTG
- 151 AGGATCAGGG AAGTCCTCGC TGGGGATGGC CCTCTTCCGT CTGGTGGAGT
- 201 TATCTGGAGG CTGCATCAAG ATTGATGGAG TGAGAATCAG TGATATTGGC
- 251 CTTGCCGACC TCCGAAGCAA ACTCTCTATC ATTCCTCAAG AGCCGGTGCT
- 301 GTTCAGTGGC ACTGTCAGAT CAAATTTGGA CCCCTTCAAC CAGTACACTG
- 351 AAGACCAGAT TTGGGATGCC CTGGAGAGGA CACACATGAA AGAATGTATT
- 401 GCTCAGCTAC CTCTGAAACT TGAATCTGAA GTGATGGAGA ATGGGGATAA
- 451 CTTCTCAGTG GGGGAACGGC AGCTCTTGTG CATAGCTAGA GCCCTGCTCC
- 501 GCCACTGTAA GATTCTGATT TTAGATGAAG CCACAGCTGC CATGGACACA
- 551 GAGACAGACT TATTGATTCA AGAGACCATC CGAGAAGCAT TTGCAGACTG
- 601 TACCATGCTG ACCATTGCCC ATCGCCTGCA CACGGTTCTA GGCTCCGATA
- 651 GGATTATGGT GCTGGCCCAG GGACAGGTGG TGGAGTTTGA CACCCCATCG
- 701 GTCCTTCTGT CCAACGACAG TTCCCGATTC TATGCCATGT TTGCTGCTGC
- 751 AGAGAACAAG GTCGCTGTCA AGGGCTGACT CCTCCCTGTT GACGAAGTCT
- 801 CTTTTCTTTA GAGCATTGCC ATTCCCTGCC TGGGGCGGCC CCCTCATCGC

FIG. 19b

- 851 GTCCTCCTAC CGAAACCTTG CCTTTCTCGA TTTTATCTTT
- 901 TTCCGGATTG GCTTGTGTT TTCACTTTTA GGGAGAGTCA TATTTTGATT
- 951 ATTGTATTTA TTCCATATTC ATGTAAACAA AATTTAGTTT TTGTTCTTAA
- 1001 TTGCACTCTA AAAGGTTCAG GGAACCGTTA TTATAATTGT ATCAGAGGCC
- 1051 TATAATGAAG CTTTATACGT GTAGCTATAT CTATATATAA TTCTGTACAT
- 1151 GCACTGTGCT AATAACAGTG CATATTCCTT TCTATCATTT TTGTACnGTT
- 1201 TGCTGTACnA nAAATCTGGT nTTGCTmTTm nACTGTTAGG AAGAATTAnC
- 1251 ATTTCATTCT TCTCTAGCTG GTGGTTtCAC gGTGgCCAGG
- 1301 GTCCAAAGGA AGACGTGTtG GCAATAGTtn GGGCCCTCCG ACAAGCCCCC
- 1351 TCTGCCGCCT CCCCACAGCC GCTCCAnGGG GTGGCTGGAG AaCGGGTGGG
- 1401 CGGCTGGAGA CCATGCCAGA GCGCCGTGAG TTCTCAGGGC TCCTGCCTTC
- 1451 TGTCCTGGTG TCACTTACTG TTTCTGTtCA GGGAGAGCAG CGGGGCGAAG
- 1501 CCCAGGCCCC TTTTCACTCC CTCCATCAAG AATGGGGATC ACAGAGACAT
- 1551 TCCTCCGAGC CGGGGAGTTT CTTTCCTGCC TTCTTCTTTT TGCTGTTGTT
- 1601 TCTAAACAAG AATCAGTCTA TCCACAGAGA GTCCCACTGC CTCAGGTTCC
- 1651 TATGGCTGGC CACTGCACAG AGCTCTCCAG CTCCAAGACC
- 1701 AAGCCCTGGA GCCAACTGCT GCTTTTTGAG GTGGCACTTT

FIG. 19b-2

#### 36/36

17	751	TATTCCCACA	CCTCCACAGT	TCAGTGGCAG	GGCTCAGGAT
<b>ጥጥርርጥ</b> ር	CCTC	•			

- 1801 TGTTTTCCTT TCTCACCGCA GTCGTCGCAC AGTCTCTCTC TCTCTCTCCC
- 1851 CTCAAAGTCT GCAACTTTAA GCAGCTCTTG CTAATCAGTG TCTCACACTG
- 1901 GCGTAGAAGT TTTTGTACTG TAAAGAGACC TACCTCAGGT TGCTGGTTGC
- 1951 TGTGTGGTTT GGTGTGTTCC CGCAAACCCC CTTTGTGCTG
  TGGGGCTGGT
- 2001 AGCTCAGGTG GGCGTGGTCA CTGCTGTCAT CAGTTGAATG GTCAGCGTTG
- 2051 CATGTCGTGA CCAACTAGAC ATTCTGTCGC CTTAGCATGT TTGCTGAACA
- 2101 CCTTGTGGAA GCAAAAATCT GAAAATGTGA ATAAAATTAT TTTGGATTTT

#### 2151 GTAAAAAAA AAAAAAA

FIG. 19b-3

## **PCT**

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NL et al.

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(NL). KOOL, Marcel [NL/NL]; Van Walbeeckstraat 52-I, NL-1058 CV Amsterdam (NL).

- (74) Agent: SMULDERS, Th., A., H., J.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).
- (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(54) Title: A FAMILY OF ORGANIC ANION TRANSPORTERS, NUCLEIC ACIDS ENCODING THEM, CELLS COMPRISING THEM AND METHODS FOR USING THEM

#### (57) Abstract

The present invention provides a novel family of organic anion transporters of which until now only one member was known. The family includes multispecific organic anion transporters related to the canalicular multispecific organic anion transporter. The cDNA encoding the latter is also provided. The rat and human cDNA are exemplified. Uses of nucleic acids based on this gene family and of cells comprising such nucleic acids as well as vectors comprising sequences thereof are also disclosed especially in the area of gene therapy.

Inter onal Application No PCT/NL 97/00079

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/12 C071 A61K48/00 C12Q1/68 C07K14/705 According to International Patent Classification (IPC) or to both national classification and IPC B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K C12N IPC 6 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practical, search terms used) C. DOCUMENTS CONSIDERED TO BE RELEVANT Relevant to claim No. Citation of document, with indication, where appropriate, of the relevant passages 1-6,20, MAYER R ET AL: "Expression of the MRP X 21 gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes." J CELL BIOL, OCT 1995, 131 (1) P137-50, UNITED STATES, XP000608636 see the whole document 1-6,20, COLE SP ET AL: "Overexpression of a X 21 transporter gene in a multidrug-resistant human lung cancer cell line [see comments] " SCIENCE, DEC 4 1992, 258 (5088) P1650-4, UNITED STATES, XP002017513 see the whole document -/--Patent family members are listed in annex. X I Further documents are listed in the continuation of box C. \* Special categories of cited documents : "I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the "A" document defining the general state of the art which is not considered to be of particular relevance. invention "E" earlier document but published on or after the international "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to filing date involve an inventive step when the document is taken alone "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu-"O" document referring to an oral disclosure, use, exhibition or ments, such combination being obvious to a person skilled in the art. other mean \*P\* document published prior to the international filing date but later than the priority date claimed "&" document member of the same patent family Date of mailing of the international search report Date of the actual completion of the international search **2** 1. 10. 97 10 July 1997 **Authorized officer** Name and mailing address of the ISA Europeen Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Riswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

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Intel onal Application No PCT/NL 97/00079

		PCT/NL 97/00079
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ategory *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZAMAN GJ ET AL: "The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump." PROC NATL ACAD SCI U S A, SEP 13 1994, 91 (19) P8822-6, UNITED STATES, XP002017514	1-7, 11-17, 20,21
,	see the whole document	8-10
	WO 94 10303 A (UNIV KINGSTON) 11 May 1994 see claims 1-80; figures SEQ.1,2	1-22
,	WO 92 12987 A (US) 6 August 1992 see page 32, line 26 - page 33, line 22	8-10
•	KITAMURA T ET AL: "Defective ATP-dependent bile canalicular transport of organic anions in mutant (TR-) rats with conjugated hyperbilirubinemia." PROC NATL ACAD SCI U S A, MAY 1990, 87 (9) P3557-61, UNITED STATES, XP002017515 cited in the application see the whole document	1-6
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,x	BUCHLER M ET AL: "cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats."  J BIOL CHEM, JUN 21 1996, 271 (25) P15091-8, UNITED STATES, XP002017517 see the whole document	1-6,20,
,x	PAULUSMA CC ET AL: "Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene." SCIENCE, FEB 23 1996, 271 (5252) P1126-8, UNITED STATES, XP002017518 see the whole document	1-6,20, 21

1

1 rational application No.

PCT/NL 97/00079

Boxi	Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)
This Inte	rnational Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
1. X 2.	Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely: Remark: Although claim 23 and claims 16-18, partially as far as they concern an in vivo method, are directed to a method of treatment of the human/aninmal body, a search has been carried out based on the alleged effects of the composition.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.	Claims Nos.:  because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)  ternational Searching Authority found multiple inventions in this international application, as follows:
	ee continuation-sheet
1.	As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.	As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.	As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. [	No required additional search fees were timely paid by the applicant. Consequently, this international Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.;  1-24 all partially (Subject 1.)
Ren	The additional search fees were accompanied by the applicant's protest.  No protest accompanied the payment of additional search fees.

International Application No. PCT/NL 97 00079

### FURTHER INFORMATION CONTINUED FROM PCT/ISAL 10

Subject 1: Claims 1-24 (partially): the canicular multispecific organic anion transporter cMOAT protein and gene.

Subject 2: Claims 1-24 (partially): the multidrug resistant-associated protein MRP3 protein and gene.

Subject 3: Claims 1-24 (partially): the multidrug resistant-associated protein MRP4 protein and gene.

Subject 4: Claims 1-24 (partially): the multidrug resistant-associated protein MRP5 protein and gene.

BNSDOCID: <WO\_\_\_\_\_9731111A3\_l\_>

Information on patent family members

Ints. .ional Application No PCT/NL 97/00079

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